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**13. ABSTRACT** *(Maximum 200 words)*

No significant improvements for treatment of metastatic breast cancer have been developed in the last 20 years and the prognosis for women with this disease remains poor. Progress in understanding the immune response, however, has led to renewed enthusiasm for immune-based anti-cancer therapies. Previously, we demonstrated that tumor cell-based vaccines expressing MHC class II and B7.1 (CD80) molecules reduced experimental (i.v.-induced) and established spontaneous metastatic disease, by activating tumor-specific $CD4^+$ T-lymphocytes. We now demonstrate, using the mouse 4T1 mammary carcinoma, that a novel immunotherapy consisting of the cytokine IL-12 and SEB superantigen combined with the previously described cell-based vaccine produces an even greater reduction in spontaneous metastatic disease and significant extension of mean survival time in two distinct immunotherapeutic regimens. The therapeutic effect is particularly noteworthy because: 1) spontaneous metastatic cancer by 4T1 progresses similarly in comparison to human metastatic mammary cancer, 2) our post-operative model demonstrates that early metastatic lesions are primarily responsible for morbidity, 3) the metastatic disease is extensive prior to initiation of immunotherapy in both regimens, and 4) multiple effector cell populations, including T and NK cells, and anti-angiostatic factors are likely to mediate the anti-tumor effect.

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Bobbi A. Pulaski 7-20-99

PI - Signature Date

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## Introduction

As a result of recent discoveries/advances in immunology and molecular cloning, many novel immunotherapeutic strategies for the treatment of cancer are being developed (1-3). Most of these strategies are focused on eliminating primary tumors, which are frequently successfully treated by conventional methods, such as surgery. In contrast, very few immunotherapeutic approaches are targeting disseminated metastatic disease, for which there are frequently no effective conventional therapies. Development of therapies for the treatment of metastatic disease is complicated by the shortage of adequate animal models for spontaneously metastatic cancers. We recently described a novel cell-based vaccine for the therapy of metastatic disease and tested it using the mouse 4T1 mammary carcinoma. The 4T1 tumor shares many characteristics with its human counterpart, and hence is an excellent model system for testing experimental immunotherapies. In addition to its parallels with human disease, 4T1 cells are resistant to the drug 6-thioguanine (4), enabling one to precisely quantify the number of micro-metastatic cells in the lungs, lymph nodes (LN), livers, blood, and brains of individual animals (5).

The cell-based vaccines consist of tumor cells transfected with syngeneic major histocompatability complex (MHC) class II and B7.1 co-stimulatory molecule genes and were designed to enhance activation of tumor-specific $CD4^+$ T lymphocytes via improved presentation of tumor-encoded class II-restricted epitopes. Although $CD8^+$ T lymphocytes have been traditionally the focus of immunotherapy approaches, accumulating results have demonstrated that $CD4^+$ T lymphocytes play a critical role in effective anti-tumor immunity (6-9). While our vaccine showed significant reduction of established, spontaneous metastatic tumor, the anti-tumor response was limited to small burdens of metastatic cells and did not completely eliminate metastases (5). We now report on more advanced "second-generation" cell-based vaccines that are significantly more effective than the original vaccine for the treatment of spontaneous 4T1 metastatic mammary cancer. The new vaccines incorporate a gene encoding the bacterial toxin, *Staphylococcal aureus* enterotoxin B (SEB), and the systemic administration of IL-12. SEB is a superantigen (sAg) that when complexed with MHC class II molecules on antigen presenting cells (APC) is a potent polyclonal activator of $CD4^+$ T lymphocytes (10, 11). IL-12 is a cytokine that favors $T_{h1}$ lymphocytes and NK cell development, stimulates anti-angiogenic chemokines (12, 13), and reduces tumor burden in numerous mouse tumor systems (14-16). We reasoned that addition of SEB and/or IL-12 to the MHC class II/B7.1 vaccine will provide additional activation signals to the $CD4^+$ T cells that have been activated in an antigen-specific fashion by the MHC class $II^+$ $B7.1^+$ vaccinating cells.

## Body

Technical Objective 1: Generate mammary tumor transfectants which can more effectively and directly present tumor antigen to $CD4^+$ T-cells.

The generation of transfectants in addition to those previously listed in the 1998 Annual Summary Report (ASR) (In summary..., page 11) has been discontinued. While the generation of double transfectants was originally planned, it will not be necessary to complete this task since mixing transfectants appears to be effective, as will be demonstrated in technical objective #3.

Technical Objective 2: Characterize the immunogenicity of the mammary tumor transfectants.

The parental 4T1 tumor and the single transfectants made in technical objective 1 have been previously characterized (1998 ASR pages 11-16). The *in vivo* tumor growth and spontaneous metastases development of the $4T1/A^d$.B7.1 double transfectants have not been evaluated because the single transfectants exhibited a wide spectrum of *in vivo* characteristics yet uniformly were effective as cell-based vaccines. This led us to believe that it would be a waste of time and resources to evaluate the *in vivo* characteristics of the double transfectants. Therefore, studies were immediately initiated using the double transfectants as a cell-based vaccine and are described in technical objective #3.

Technical Objective 3: Examine the ability of the immunogenic transfectants to prevent disease and/or eliminate established spontaneous metastases.

**Task 10: Radiation sensitivity of 4T1 tumor cells.**

Has been completed (1998 ASR page 17)

**Tasks 11-13: Therapy regimen one- Immunization of non-tumor bearing (naive) mice prior to challenge with parental tumor.**

Studies using the $4T1/A^d$ and 4T1/B7.1 transfectants in this therapy regimen were described previously (1998 ASR pages 17-18). Again, due to the success of the cell-based vaccines to reduce established wild type spontaneous metastatic disease (1998 ASR pages 18-20 and described below) this therapy regimen has been eliminated from these studies. Since our goal is to treat established spontaneous metastatic cancer, the focus of this research has been concentrated on tasks 14-16 of this proposal.

**Tasks 14-15: Therapy regimen two- Treatment of mice bearing established 4T1 primary tumor.**

Previously, we have shown that transfectants expressing MHC class II or B7.1 were able to reduce metastatic disease that had been established for 9-14 days. However, the success of this treatment was limited to small tumor burdens and did not completely eliminate spontaneous metastases (1998 ASR pages 18-20). To generate a more potent vaccine we have added a cell-based vaccine encoding *Staphylococcal aureus* enterotoxin B (SEB) and the systemic administration of IL-12. (see two manuscripts enclosed) Significant progress has been made to reduce spontaneous established metastases arising from larger tumor burdens as well as reducing the quantity of metastatic lesions. Due to the enormous quantity of data and page limitations of this progress report, the findings will be summarized briefly in the following paragraphs and the data will be referenced from the enclosed manuscripts. Some new experiments have been added to the SOW, where indicated, and are described below in more detail.

The therapeutic potential of the combination vaccines was rigorously tested by treating BALB/c mice with extensive established wild type primary and metastatic 4T1 tumors. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with $7x10^3$ wild type 4T1 tumor cells. Starting 2-3 weeks after challenge, they were given i.p. injections of irradiated transfectants and/or systemic IL-12, as indicated in the manuscripts, until the day of sacrifice. Therapy was initiated at 2-3 weeks post primary tumor challenge because spontaneous lung and LN metastases are well established by this time (Table 2 & Figure 6, 1998 ASR). At the time of sacrifice (6 weeks after the initial primary tumor challenge), primary tumor diameters of control-treated mice (ie. mice given irradiated 4T1 cells) were typically comparable to tumor diameters in transfectant-only-treated animals (ie. 4T1/SEB, 4T1/$A^d$, 4T1/B7.1, or 4T1/$A^d$.B7.1). In contrast, addition of systemic IL-12 to the therapy protocol could reduce tumor growth depending on the size of the primary tumor at the time therapy began (Table 2, manuscript entitled IL-12 synergizes...).

To assess the metastatic disease, lungs from the treated mice were removed and processed to determine the number of clonogenic tumor cells. Since this therapy will be used to treat patients with established tumor, the results of this experiment have been plotted as number of clonogenic metastatic cells in the lungs vs. tumor diameter at the start of treatment. As shown in Figure 3 (manuscript entitled Synergy of SEB...), 3/16 (18.8%) mice treated with irradiated parental cells (Figure 3A) contained $<10^4$ clonogenic lung metastases in contrast to 11/23 (47.8%) mice treated with either 4T1/SEB or 4T1/$A^d$/B7.1 (Figures 3B and 3C) and 15/21 (71.4%) mice treated with a mixture of transfectants (Figure 3D). After transforming the number of clonogenic metastases to logarithmic values and analyzing using the Tukey's Honestly Significant Difference Test

(p= 0.05) only the mixture of 4T1/SEB and 4T1/$A^d$/B7.1 (Figure 3D) treatment was significantly different from the 4T1 control-treated mice. Treatment with IL-12 alone causes some reduction in the number of clonogenic metastatic cells relative to control 4T1 treated mice (Figure 3B vs. Figure 3A, IL-12 synergizes...) however, 44% of mice (8/18) still have $>10^4$ metastatic cells in their lungs (Figure 3B). In contrast, treatment with IL-12 plus any transfectant or IL-12 plus wild type 4T1 results in dramatic reduction in the number of lung metastases in individual mice and a reduction in the number of mice with detectable metastatic cells (Figures 3D-3G) vs. mice treated with 4T1 cells alone (Figure 3A). Pooling all of the groups treated with cells plus IL-12 (Figuers 3D-3G) shows that only 21% of mice (11/53) have $>10^4$ metastastic tumor cells in the lungs following treatment (Tukey's p = 0.05).

**NEW THERAPY REGIMEN**

While the transfection of MHC class II, SEB, and B7.1 into 4T1 cells could enhance the immunogenicity of this tumor and significantly reduce established spontaneous metastatic disease, it became clear that the efficacy of the vaccine could not be evaluated accurately in the two therapy regimens described in the original grant proposal and 1998 annual report. The tumor vaccines have been tested for their therapeutic efficacy in a model system where the primary mammary tumor remained in tact. A potential problem with this model is that metastatic cells could continue to spread to the target organs. As a result, a third therapy regimen was developed in which the primary tumor was removed surgically.

In order to test the vaccines in a "surgical" model, we determined the appropriate time for removing primary tumors that would guarantee establishment of spontaneous metastases. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with $7x10^3$ wild type 4T1 tumor cells. Starting 2 weeks after challenge, their tumors were measured and surgically removed at 1 week intervals. Figure 4A (Synergy of SEB...) shows the tumor size (mm) at the time of surgery vs. the number of days the mice survived post 4T1 tumor challenge. The average survival time of 55/58 mice was 48.9 ($\pm$ 7.4) days while the remaining 3 mice, whose tumor diameter was < 3mm at the time of surgery, lived >90 days and did not succumb to metastatic cancer. Surprisingly, all mice that died from spontaneous metastatic disease showed approximately the same mean survival time regardless of the size of the primary tumor at the time of surgery. These results demonstrate that lethal metastasis occurs as early as 2 weeks after inoculation of primary tumor and that death following surgical removal of primary tumor results from outgrowth of tumor cells that metastasize early during primary tumor growth.

Based on these data, we have begun to test several combinations of immunotherapy in post-surgery mice. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7 X $10^3$ wild type 4T1 tumor cells. In the first group of surgery experiments, the tumors were allowed to grow and metastasize for 3 weeks, at which time the primary tumor burden was measured and surgically resected. The experiments in Figures 3 and 4A (Synergy of SEB...) and our previously published results (pages 13-14 1998 ASR) established that at this stage and size of primary tumor the mice have very extensive disseminated metastases. At the time of surgery, the mice were divided into treatment groups based on the sizes of their primary tumor. The average diameter of primary tumors in these groups at the time of surgery was comparable. Therapeutic injections of irradiated cells (1x$10^6$) were started 1 week after surgery (i.e. 4 weeks after initial tumor challenge) and were administered once a week for the duration of the experiment. Injections of IL-12 (1 μg per mouse) were also started 1 week after surgery and administered 3 times a week for the duration of the experiment. Figures 1A and 1B (below) shows the survival profiles. In Figure 1A, the average survival time in days for 4T1-treated, 4T1/SEB-treated, 4T1/$A^d$.B7.1-treated, and 4T1/SEB+4T1/$A^d$.B7.1-treated animals was 45.2 (±5.4), 48.3 (±6.3), 50.1 (±2.3), and 51.9 (±8.7), respectively. In Figure 1B, the average survival time in days for 4T1-treated, IL-12-treated, and 4T1+IL-12-treated animals was 40.5 (±4.0), 58.6 (±17.2), and 57.7 (±8.7), respectively. Statistical analyses using the Tukey's Honestly Significant Difference Test (p= 0.05) reveals that treatments with either 4T1/$A^d$.B7.1+4T1/SEB or IL-12 significantly increase survival for mice with established wild type metastatic disease. Interestingly, when animals were given a combination of IL-12 plus 4T1/$A^d$.B7.1+4T1/SEB as therapy, we found that the therapy itself was lethal to the

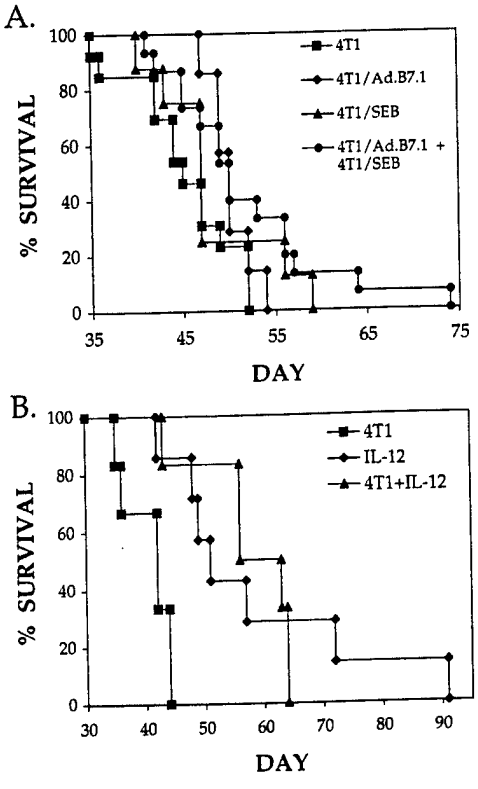


**Figure 1.** Immunotherapy reduces established wild type spontaneous metastases in a surgical regimen. A) Vaccine using a mixture of MHC class II/B7.1 and SEB transfectants increases survival. B) Vaccine using systemic IL-12 injections increases survival. Female BALB/c mice were challenged with 7x$10^3$ wild type 4T1 tumor cells. Primary tumors were measured and surgically resected 21 days post-parental tumor challenge. Mice were treated with therapy beginning on day 28 as described in text.

animals. Work is currently in progress to determine which levels of this vaccine will be safe for the animals and at the same time remain effective against metastatic disease.

**Task 16: Analysis of immune response mechanism.**

The concept of combining SEB with MHC class II and B7.1 was based on the hypothesis that SEB is a potent polyclonal activator of $CD4^+$ T lymphocytes (10, 11) and would provide additional activation signals to the $CD4^+$ T cells that have been activated in an antigen-specific fashion by the MHC class $II^+$ $B7.1^+$ vaccinating cells. Therefore, to begin understanding the mechanism of this vaccine's efficacy and demonstrate that T-cells are involved in the immune response against metastatic cancer, we tested the immunotherapy in nude mice and GK1.5 (CD4) or 2.43 (CD8) antibody-depleted animals (Figure 2 below). As shown in Figures 2C and 2D, depletion of $CD4^+$ or $CD8^+$ T cells eliminates the therapeutic effect of the MHC class $II^+$, $B7.1^+$, $SEB^+$ vaccine against spontaneous metastases, while depletion with control ascites (Figure 2B) does not. In addition, the combination vaccine does not reduce metastatic disease in BALB/c *nu/nu* mice (Figure 2F), which are deficient for T cells.

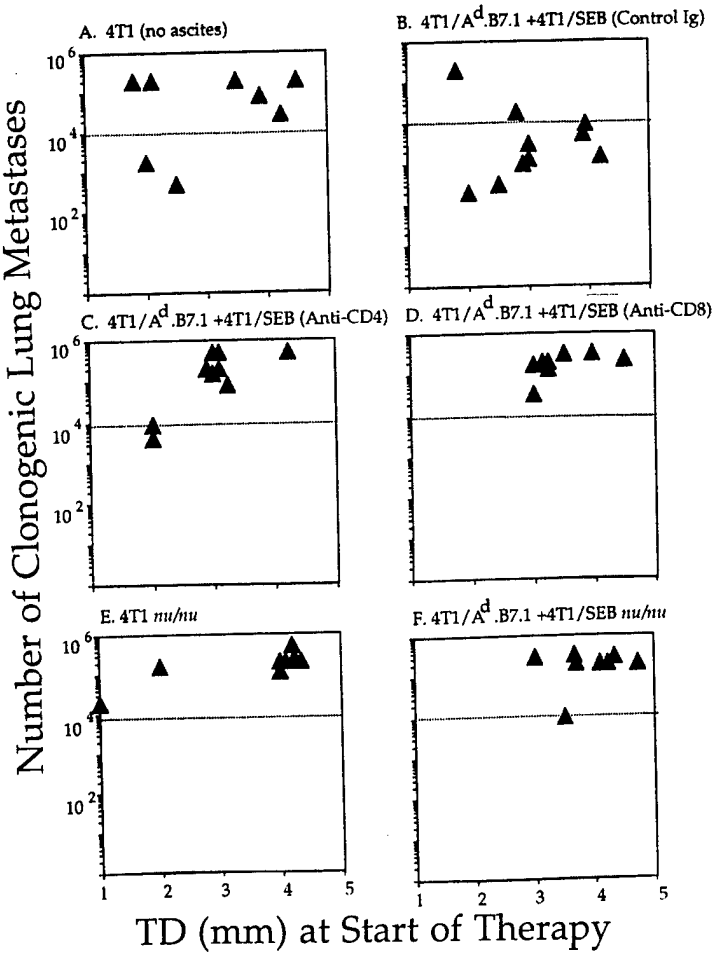


**Figure 2:** Reduction of established wild type metastases with MHC class II, B7.1, and SEB immunotherapy requires both $CD4^+$ and $CD8^+$ T cells. A-D) Naïve BALB/c mice were injected s.c. in the abdominal mammary gland with $7x10^3$ parental 4T1 cells and, beginning on day 14, treated with either (A) 4T1 parental cells (8 mice) or (B-D) a 1:1 mixture of 4T1/A$^d$.B7.1 plus 4T1/SEB. On day 8, 11, and 13 (ie. prior to the start of immunotherapy) mice were injected with either (B) control ascites (10 mice), (C) GK1.5 ascites (9 mice), or (D) 2.43 ascites (8 mice) and continued at least once a week for the duration of the experiment. E-F) BALB/c *nu/nu* mice were injected s.c. in the abdominal mammary gland with $7x10^3$ parental 4T1 cells and, beginning on day 14, treated with either (E) 4T1 parental cells (8 mice) or (F) a 1:1 mixture of 4T1/A$^d$.B7.1 plus 4T1/SEB (8 mice)

The concept of combining a cell-based immunization therapy with IL-12 was based on the hypothesis that IL-12 facilitates the development of $T_{h1}$-type $CD4^+$ T helper lymphocytes (17) that are activated by immunization with the MHC class $II^+B7.1^+$ tumor cells. These $T_{h1}$ cells would, in turn, provide "help" to tumor-specific $CD8^+$ T lymphocytes and improved anti-tumor immunity would result. To test this hypothesis and to determine the involvement of T cells, mice undergoing immunotherapy were depleted for $CD4^+$ and $CD8^+$ T cells. To our surprise, Figure 3H-3J (IL-12 synergizes...) shows that depletion of $CD4^+$ or $CD8^+$ T cells does not diminish the therapeutic effect for the 4T1 tumor.

In addition to its role in $T_{h1}$ development, IL-12 also stimulates NK cell function (18). We, therefore, analyzed the role of NK cells in mice undergoing immunotherapy. Splenocytes of BALB/c mice carrying established 4T1 metastases and having at least 2 weeks of immunotherapy were tested for pan NK, $CD4^+$, $CD8^+$, $CD3^+$, and $B220^+$ expression. The percentages of $CD4^+$, $CD8^+$, and $B220^+$ splenocytes did not change following therapy with cells and/or IL-12 (Table 1 below). In contrast, as shown in Table 3 (IL-12 synergizes...), the percentage of pan $NK^+$ splenocytes in BALB/c mice was higher in all treated mice relative to untreated control mice. To determine if NK cells are responsible for the anti-metastatic effects of the cell-based therapy on 4T1 tumor growth, therapy mice were depleted for NK cells using anti-asialo-GM1 polyclonal antibodies prior to initiation of therapy. As shown in Figure 3K (IL-12 synergizes...), therapy mice depleted for NK cells have more metastatic tumor cells in their lungs than non-NK depleted mice, however, they do not have levels of metastatic cells as high as untreated mice. NK cells, therefore, may mediate some of the therapy effect, however, other effector mechanisms are probably also involved.

| Primary Tumor | Therapy | | Percent Cells in Spleen | | |
|---|---|---|---|---|---|
| | Cells | IL-12 | $CD3^+CD4^+$ | $CD3^+CD8^+$ | $B220^+$ |
| none | --- | --- | 28.2 ± 0.5 | 15.9 ± 1.8 | 25.6 ± 6.0 |
| 4T1 | 4T1 | --- | 19.8 ± 4.1 | 9.7 ± 3.4 | 20.7 ± 2.9 |
| | --- | IL-12 | 14.1 ± 2.4 | 7.3 ± 1.1 | 22.0 ± 0.2 |
| | 4T1 | IL-12 | 19.3 ± 3.2 | 10.1 ± 2.1 | 25.4 ± 5.3 |

**Table 1:** Mice carrying established 4T1 metastases and treated with IL-12 and/or cell transfectants have similar levels of $CD4^+$, $CD8^+$, and $B220^+$ splenocytes.

While neither T nor NK cells were exclusively responsible for the therapeutic effect, these cell populations could have been functioning cooperatively to diminish growth of metastatic cells. To test this hypothesis, beige/nude/XID mutant mice, which are deficient

for NK and T cells, were challenged with 4T1 wild type tumor and given immunotherapy treatments. As shown in Figure 4 (IL-12 synergizes...), therapy with IL-12 ± cells results in fewer metastatic cells in the lungs as compared to therapy with 4T1 alone, however, the reduction is not as dramatic as that seen in fully immunocompetent mice (see Figure 3, IL-12 synergizes...). Beige/nude/XID mice, therefore, respond to the therapy (two-tailed student's t-test, $p < 0.003$), however, the response is not as great as that seen in immunocompetent mice, suggesting that NK and T cells are partially responsible for the therapy effect.

**NEW MECHANISTIC STUDIES**

Recent studies indicate that IL-12 and its downstream mediator IFN-γ may regulate tumor growth by stimulating anti-angiogenic chemokines including Monokine Induced by IFN-γ (Mig) and IFN-γ Inducible Protein 10 (IP-10) (12, 13, 19). To determine if our combination therapy involves Mig and/or IP-10, RNA was prepared from the lungs of tumor-bearing therapy mice, reverse-transcribed, and PCR amplified using Mig-specific and IP-10-specific PCR primers. Although IP-10 mRNA was not expressed in the lungs, Mig mRNA was present in the lungs of 4T1 tumor-bearing mice receiving immunotherapy. Mig mRNA was detected as early as 4 hours after initiation of therapy and throughout the 21-day duration of therapy (Figure 5C, IL-12 synergizes...). Control untreated, tumor-free mice did not express Mig in their lungs (Figure 5A).

To determine if the tumor cells synthesize Mig and therefore produce the Mig detected in the lungs, 4T1 cells were cultured with IFN-γ for 2 hours *in vitro* and Mig and β-actin expression analyzed by reverse PCR. As shown in Figure 6 (IL-12 synergizes...), IFN-γ induces expression of Mig in 4T1 tumor cells.

**Future Directions**

**Tasks 14-15:** Combine all cellular therapies with IL-12 in the surgery regimen

**Task 16:** Examine the mechanism further

1) Examine effects of immunotherapy in knockout mice
    1.1) IFN-γ knockout mice
    1.2) CXCR3 (Mig chemokine receptor) knockout mice, if available
2) Isolate T cells from lungs of mice undergoing therapy
    2.1) evaluate cell surface markers
    2.2) examine funtionality in $^{51}Cr$ release assays
    2.3) attempt adoptive transfer experiments

## References


1. Sogn, J. Tumor immunology: the glass is half full. Immunity, *9:* 757-763, 1998.
2. Lattime, E.C., and Gerson, S.L. (eds.), Gene Therapy of Cancer. San Diego: Academic Press, 1998.
3. Blankenstein, T., Cayeux, S., and Qin, Z. Genetic approaches to cancer immunotherapy. Rev. Phys. Biochem. Pharm., *129:* 1-49, 1996.
4. Aslakson, C.J., and Miller, F.R. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res., *52:* 1399-1405, 1992.
5. Pulaski, B.A., and Ostrand-Rosenberg, S. Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines. Cancer Res., *58:* 1486-1493, 1998.
6. Hung, K., Hayashi, R., Lafond-Walker, A., Lowenstein, C., Pardoll, D. and Levitsky, H. The central role of CD4+ T cells in the antitumor immune response. J. Exp. Med., *188:* 2357-2368, 1998.
7. Pardoll, D., and Topalian, S. The role of CD4+ T cell responses in antitumor immunity. Curr. Opin. Immunol., *10:* 588-594, 1998.
8. Ostrand-Rosenberg, S. Tumor immunotherapy: the tumor cell as an antigen presenting cell. Curr. Opin. Immunol., *6:* 722-727, 1994.
9. Greenberg, P. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. Adv. Immunol., *49:* 281-355, 1991.
10. Marrack, P., Blackman, M., Kushnir, E., and Kappler, J. The toxicity of staphylococcal enterotoxin B in mice is mediated by T cells. J. Exp. Med., *171:* 455-464, 1990.
11. Hsieh, C., Macatonia, S., Tripp, C., Wolf, S., O'Garra, A., and Murphy, K. Development of Th1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science, *260:* 547-549, 1993.
12. Coughlin, C. Salhany, K., Gee, M., LaTemple, C., Kotenko, S., Ma, X., Gri, G., Wysocka, M., Kim, J., Liu, L., Liao, F., Farber, J., Pestka, SS., Trinchieri, G., and Lee., W. Tumor cell responses to IFN-gamma affect tumorigenicity and response to IL-12 therapy and antiangiogenesis. Immunity, *9:* 25-34, 1998.
13. Kanegane, C., Sgdari, C., Kanegane, H., Teruya-Feldstein, J., Yao, L., Gupta, G., Farber, J., Liao, L., and Tosato, G. Contribution of the CXC chemokines IP-10 and Mig to the antitumor effects of IL-12. J. Leukoc. Biol., *64:* 384-392, 1998.

14. Brunda, M., Luistro, L., Warrier, L., Wright, R., Hubbard, B., Murphy, M., Wolf, S., and Gately, M. Anti-tumor and anti-metastatic activity of IL-12 against murine tumors. J. Exp. Med., *178:* 1223-1230, 1993.
15. Tahara, H., Zeh, H., Storkus, W., Pappo, I., Watkins, C., Gubler, U, Wolf, S., Robbins, P., and Lotze, M. Fibroblasts genetically engineered to secrete interleukin-12 can suppress tumor growth and induce antitumor immunity to a murine melanoma *in vivo*. Can. Res., *54:* 182-189, 1994.
16. Tahara, H., Zitvogel, L., Storkus, W., Zeh, H., McKinney, T., Schreiber, R., Gubler, U, Robbins, P., and Lotze, M. Effective eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector. J. Immunol., *154:* 6466-6474, 1995.
17. Herman, A., Kappler, J.W., Marrack, P., and Pullen, A.M. Superantigens: mechanism of T-cell stimulation and role in immune responses. Ann. Rev. Immunol. *9:* 745-772, 1991.
18. Brunda, M. Interleukin-12. J. Leukoc. Biol., *55:* 280-288, 1994.
19. Tannenbaum, C., Tubbs, R., Armstrong, D., Finke, J., Bukowski, R., and Hamilton, T. The CXC chemokines IP-10 and Mig are necessary for the IL-12-mediated regression of the mouse RENCA tumor. J. Immunol., *161:* 927-932, 1998.

**Appendices**

1). Key Reasearch Accomplishments:

- Treatment of tumor-bearing mice with transfectants expressing MHC Class II, B7.1, and SEB reduces established wild type metastatic cancer.
- Mice carrying established 4T1 mammary carcinoma metastases have reductions in metastatic disease following treatment with IL-12 plus 4T1 transfectants (4T1/$A^d$ and 4T1/B7.1) or wild type tumor.
- Combination IL-12 and cellular therapy affects growth of small primary solid tumors.
- Development of 4T1 model in new surgical therapy regimen.
- Treatment of established wild type metastases in post-operative mice with transfectants expressing MHC Class II, B7.1, and SEB increases survival.
- Treatment of established wild type metastases in post-operative mice with IL-12 increases survival.
- Reduction of established wild type metastases with MHC class II, B7.1, and SEB immunotherapy requires both $CD4^+$ and $CD8^+$ T cells
- Effects of IL-12 immunotherapy is not exclusively dependent on $CD4^+$, $CD8^+$, or NK cells.

- 4T1 tumor cells are induced by IFN-γ to express the chemokine Mig.

2. Reportable Outcomes:

-Manuscripts:

1. IL-12 synergizes with MHC class II/B7.1-cell based vaccines to stimulate immune effectors and anti-angiostatic mechanisms that reduce established metastatic disease (submitted to Cancer Research, 7/99)
2. Synergy of SEB superantigen, MHC Class II, and CD80 in immunotherapy of advanced spontaneous metastatic breast cancer (submitted to Cancer Research, 2/99)

-Abstracts/Presentations:

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# IL-12 synergizes with MHC class II/B7.1-cell based vaccines to stimulate immune effectors and anti-angiostatic mechanisms that reduce established metastatic disease[1]

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[4] The following abbreviations are used: $T_{h1}$, T helper type 1 lymphocytes; 4T1/$A^d$/B7.1, 4T1 tumor cells transfected with the I-$A^d$ and CD80 genes; melF10/$A^b$/B7.1, melF10 tumor cells transfected with the I-$A^b$ and CD80 genes

**Abstract**

Because they are difficult to treat, animal models of wide-spread, established metastatic cancer are rarely used to test novel immunotherapies. Two such mouse models are used in this report to demonstrate the therapeutic efficacy and to probe the mechanisms of a novel combination immunotherapy consisting of the cytokine IL-12 combined with a previously described MHC class II, CD80 expressing cell-based vaccine. BALB/c mice with 3 week established primary 4T1 mammary carcinomas of up to 6 mm in diameter and with extensive, spontaneous lung metastases have a significant reduction in lung metastases following a 3 week course of immunotherapy consisting of weekly injections of the cell based vaccine plus 3 times/week injections of IL-12. C57BL/6 mice with 7 day established intravenous B16 melF10 lung metastases show a similar response following immunotherapy with IL-12 plus a B16-based MHC class II, CD80-expressing cell-based vaccine. In both systems the combination therapy of cells plus IL-12 is significantly more effective than IL-12 or the cellular vaccine alone, although in the 4T1 system optimal activity does not require MHC class II and CD80 expression in the vaccine cells. The cell-based vaccines were originally designed to specifically activate tumor-specific $CD4^+$ T lymphocytes and thereby provide helper activity to tumor-cytotoxic $CD8^+$ T cells and IL-12 was added to the therapy to facilitate $T_{h1}$ differentiation. In vivo depletion experiments for $CD4^+$ and $CD8^+$ T cells and NK cells and tumor challenge experiments in *beige/nude/XID* immunodeficient mice demonstrate that the therapy effect is not exclusively dependent on a single cell population, suggesting that T and NK cells are acting synergistically to optimize the response. IL-12

reverse PCR experiments demonstrate that Mig is present in the lungs of therapy mice and is mostly likely synthesized by the tumor cells because both 4T1 and melF10 tumors cells produce Mig following a 2 hour in vitro incubation with IFN-γ. These results demonstrate that the combination therapy of systemic IL-12 and a cell-based vaccine is a potent agent for the treatment of advanced, disseminated metastatic cancers in experimental mouse models. Mechanistically, multiple effector cell populations including T and NK cells, and angiostatic factors are likely to mediate the effect.

## INTRODUCTION

Many of the recently explored immunotherapy strategies for the treatment of cancer have focused on the improved activation of tumor-specific immunity. For example, administration of IL-12, a cytokine that favors $T_{h1}$[4] and NK cell development and stimulates anti-angiogenic chemokines (1, 2) reduces tumor burden in numerous mouse tumor systems (3-5). Likewise, the treatment of mice with established primary and/or metastatic tumor with irradiated immunogenic tumor cells constitutively expressing MHC class I molecules and transfected/transduced with the costimulatory molecule CD80, reduces primary tumor mass and/or metastatic tumor load (6, 7). This latter approach is based on the premise that the genetically engineered tumor cells present both antigen-specific and costimulatory T cell activation signals to the relevant $CD8^+$ T lymphocytes.

Another immunotherapeutic strategy aimed at specifically improving the generation of tumor-specific $CD4^+$ T lymphocytes uses autologous tumor cells transfected with syngeneic MHC class II genes plus CD80 costimulatory molecule genes as cell-based vaccines for the treatment of mice with established primary and metastatic cancer. This therapy is based on the premise that enhanced generation of $CD4^+$ tumor-specific T helper lymphocytes will facilitate $CD8^+$ T cell activation and promote stable, long term immune memory against recurrence of primary tumor and/or outgrowth of micrometastases (8, 9). Treatment with MHC class II-transfected cell based vaccines has yielded impressive reductions in solid tumor mass (10, 11) and in metastatic disease (12).

In an attempt to generate a more potent anti-tumor effect, IL-12 and CD80

therapies have been combined to target the activation of $CD8^+$ T cells. Because in vitro studies have shown that IL-12 plus CD80 produces optimal T cell proliferation and IFN-γ production (13, 14) as well as stimulating a primary anti-tumor response in vitro (15), it is not surprising that IL-12 and CD80 synergize to give significant tumor regression of established primary tumor as well as inducing immunological memory against recurrence of primary tumor (16, 17). Although a principal function of IL-12 is its ability to promote $CD4^+$ $T_{h1}$ differentiation, surprisingly, IL-12 therapy has not previously been combined with other therapies that specifically target the activation of tumor-specific $CD4^+$ T cells. To test the potential synergistic effect of IL-12 plus $CD4^+$-targeting approaches, we have combined systemic IL-12 therapy with immunization using MHC class II/CD80 genetically modified tumor cells for the treatment of metastatic disease. Previous in vivo studies testing IL-12 therapy have used mouse tumor models consisting of either solid, subcutaneous primary tumors, or very early metastases induced by intravenous injection of malignant cells (3, 16-21). Although these model systems provide some insight into the potential role of therapeutic agents in the treatment of cancer, they are not realistic clinical situations in which larger metastatic tumor loads are likely to be encountered and for which more effective treatments are most needed.

To more closely approximate clinical cancer, we have used two mouse tumor systems that display more of the characteristics of human tumors. The 4T1 mammary carcinoma tumor is a very poorly immunogenic and highly malignant tumor that rapidly and spontaneously metastasizes to lymph nodes, lung, liver, brain, and blood following growth of the primary tumor in the mammary fat pad (12, 22). This disease progression closely parallels human breast cancer and makes the 4T1 tumor an excellent model for human disease (12) and a rigorous animal model of advanced metastatic disease for

testing potential therapies.

As a second model system, we have used the B16-derived melF10 melanoma (23). This tumor is also very poorly immunogenic and highly malignant, and metastasizes immediately to the lung when inoculated intravenously. Similar to approximately 15% of human cancers, melF10 has markedly reduced levels of MHC class I molecules. This phenotype probably contributes to its reduced immunogenicity and heightened tumorigenicity. Many investigators have used the melF10 tumor as an experimental model, however, most studies use very early established lung metastases (e.g. ≤ 3 days post i.v. inoculation). We have used longer term established melF10 lung metastases (therapy begins on day 7 after i.v. inoculation) to more rigorously test the combination vaccine. For both tumors, the combined therapy is significantly more effective than either therapy alone, and appears to be mediated by multiple independent effector mechanisms including T lymphocytes, NK cells, and anti-angiostatic factors.

## MATERIALS AND METHODS

**Cells and transfectants:** MelF10 is a high metastatic variant of the C57BL/6-derived B16 melanoma (23). 4T1 is a spontaneously metastatic, poorly immunogenic BALB/c-derived mammary carcinoma (22). Culture conditions for both tumors have been previously described (12, 24). Generation and characterization of 4T1 transfectants expressing I-$A^d$ and CD80 and B16melF10 transfectants expressing I-$A^b$ and CD80 have been previously described (12, 24).

**Mice:** Mice were purchased from The Jackson Laboratory (Bar Harbor, Me) or bred in the UMBC Animal Facility from breeding pairs purchased from The Jackson Laboratory. Experiments using the 4T1 mammary carcinoma or melF10 melanoma were performed in female BALB/c mice and C57BL/6 male or female mice, respectively. All mice were between 6 weeks and 6 months in age.

**Tumor challenges and metastases assays:** Tumorigenesis and metastasis formation by the melF10 (24) and 4T1 (12) tumors were performed as previously described. Briefly, for experimental metastases $10^5$ melF10 cells/100 µl/mouse were inoculated intravenously (i.v.) in the tail vein of C57BL/6 mice on day 0 and mice sacrificed 3-4 weeks later and their lungs weighed and/or tumor nodules counted. For spontaneous metastases 7 X $10^3$ 4T1 cells/50 µl/mouse were inoculated in the abdominal mammary fat pad of BALB/c female mice on day 0 and mice sacrificed 6 weeks later and the number of clonogenic metastatatic cells in the lungs assessed by growth in medium supplemented with 6-thioguanine (12). Mice carrying tumors were closely followed for symptoms of pain and distress and were sacrificed when they became moribund. Based on previous studies (12) 4T1-bearing mice with $\leq$10,000 clonogenic metastatic 4T1 cells in their lungs are considered "responder" mice. All animal procedures were approved by the UMBC IACUC and are consistent with the NIH guidelines for the humane treatment of laboratory animals.

**Antibodies:** Protein A or Protein G purification of MHC class II-specific mAbs 3JP (I-$A^b$; (25)), MKD6 (I-$A^d$; (26)), MHC class I-specific mAbs 20-8-4 (H-$2K^b$; (27)), 28-14-8 (H-

$2D^b$; (27)), CD4 (GK1.5; (28)), and CD8 (2.43; (29)) was as previously described (12, 24). Fluorescently coupled CD3, CD4, CD8, NK1.1, B220, and pan NK (DX5) mAbs were purchased from Pharmingen.

**In vivo depletions for $CD4^+$ and $CD8^+$ T cells and NK cells:** Depletions of $CD4^+$ and $CD8^+$ T cells were performed as previously described (11). Briefly, mAbs to $CD4^+$ (GK1.5) and $CD8^+$ (2.43) T lymphocytes were prepared as ascites fluid in nude mice and had titers of greater than or equal to 1/80,000 as measured by immunofluorescence. Mice were inoculated intraperitoneally (i.p.) with 100 µl (GK1.5) or 150 µl (2.43) of ascites fluid mixed with an equal volume of PBS on days -6, -3, and -1 before tumor challenge, and then once a week during tumor growth. Splenocytes of all depleted mice were checked by immunofluorescence for depletion at the conclusion of the experiment. Mice depleted for $CD4^+$ or $CD8^+$ T cells had $\leq$10% or 1% of $CD4^+$ T cells or $CD8^+$ T cells, respectively.

BALB/c mice were depleted for NK cells by i.p. inoculation with 40 µl anti-asialo-GM1 antiserum (Wako Pharmaceuticals; reconstituted as directed by manufacturer) on day -4 before therapy began and twice a week while therapy was continued until the day of sacrifice. To ascertain NK depletions, one day after depletion, mice were poly IC-boosted (1 mg poly IC i.p.) and their spleens tested by $^{51}Cr$ release assay one day later. At E:T ratios of 100:1, 50:1, and 25:1, depleted mice yielded 12%, 1%, and 0% cytotoxicity, respectively, vs. control rabbit antibody treated mice which yielded 22%, 8%, and 6% cytotoxicity, respectively, against YAC-1 targets.

**NK assays:** NK assays were performed as previously described (30) using P815 cells

as NK-resistant targets and YAC-1 cells as NK-susceptible targets. Briefly, target cells (up to $10^7$) were radiolabeled with $^{51}Cr$ for 1.5 hours and chased for 30 minutes at 37°C in a volume of 0.5 ml. Labeled targets (5 X $10^4$/well) were incubated with splenocytes (effectors) at ratios ranging from 100:1 to 6.25:1 in a final volume of 200 µl/well in 96-well plates. Cells were incubated at 37° C for approximately 4 hours, and supernatants harvested and counted in a Wallac minigamma counter. Percent chromium release was calculated as ([experimental cpm - spontaneous cpm]/[total cpm - spontaneous cpm]) X 100%. Mice were induced for NK activity by i.p. inoculation of 100 µl poly IC 24 hours prior to removal of spleens.

**IL-12 and cell therapy:** IL-12 therapy consisted of i.p. inoculations of 1 µg/200 µl/mouse 3 times per week for 3 weeks. Cell therapy consisted of i.p. inoculations of $10^6$ irradiated (5000 rads) melF10, 4T1, melF10 transfectants, or 4T1 transfectants once a week for 3 weeks. Therapy for 4T1 and melF10 experiments were started at 21 and 7 days after wild type tumor challenge, respectively.

***In Vitro* and *In Vivo* MIG Detection by Reverse PCR:** 4T1 and MelF10 cell lines were induced *in vitro* with 100 U/ml IFN–γ for 2h. Naive mice were injected with 4T1 parental tumor and treated with immunotherapy as described above. Lung tissue was removed at the indicated times after the start of immunotherapy treatment. RNA was isolated from all samples by using RNA-STAT and DNAse treated with RQ1 RNase-free DNase as directed by the manufacturer. cDNA was generated using oligo dT(12-18) primers and MMLV reverse transcriptase. PCR amplification for β-actin and Mig was performed

using the following primer pairs:

MuMig primers: forward (5'GATCAAACCTGCCTAGA3');

reverse (5'CTTGAACGACGACGAC3')

β-actin primers: forward (5'GTCCCTGTATGCCTCTG3');

reverse (5'ATGAGGTAGTCTGTCAGGT3')

**Statistical Analyses:** To determine the statistical significance of the data, the student's t-test for unequal variances (Microsoft Excel version 5.0) and Tukey's Honestly Significant Difference Test, at a p-value equal to 0.05, were performed as indicated.

## RESULTS

**Treatment of mice with established B16melF10 lung metastases with transfected tumor cells plus IL-12 reduces metastatic disease.** To test the combination therapy of IL-12, MHC class II, and CD80, we have transfected the melF10 tumor with syngeneic MHC class II genes ($Aa^b$ and $Ab^b$ genes encoding the I-$A^b$ class II molecule) plus the CD80 gene. Transfectants were screened by indirect immunofluorescence for class II and/or CD80 expression and cloned by limiting dilution. Figure 1 shows the flow cytometry profiles of melF10 cells transfected with MHC class II genes (melF10/$A^b$), CD80 gene (melF10/B7.1), or both class II plus CD80 (melF10/$A^b$/B7.1) and stained with mAbs for MHC class I, class II, or CD80. Wild type

melF10 and the transfectant cells have relatively low levels of endogenously encoded MHC class I molecules (H-2K$^b$ and H-2D$^b$; 20-8-4 and 28-24-8 mAbs, panels i-l and m-p, respectively). I-A$^b$ and CD80 are only expressed on those cell lines carrying these transgenes (panels b and d; g and h, respectively).

Because our goal is to generate more effective therapeutic strategies, the therapeutic efficacy of the melF10 transfectants plus IL-12 was tested in mice with established lung metastases. Syngeneic C57BL/6 mice were inoculated i.v. in the tail vein with $10^5$ wild type melF10 cells. Following i.v. injection the melF10 cells migrate to the lungs within hours, and mice die from metastatic lung tumor within 3-4 weeks (Clements and Ostrand-Rosenberg, unpublished). On day 7 after inoculation of wild type tumor, immunotherapy was started. Mice were given 1 injection/week of $10^6$ irradiated tumor cells or transfectants and 3 injections/week of 1 μg of IL-12 per mouse. Therapy was continued for 3 weeks and mice were then sacrificed and the lungs removed, visually inspected, and weighed. Figure 2 shows the lungs of treated mice from one experiment and table 1 shows the quantitative results of 3 experiments in which tumor-bearing mice were treated with IL-12 ± melF10, melF10/A$^b$, melF10/B7.1, or melF10/A$^b$/B7.1 cells. In all experiments the greatest reduction in lung metastases was in mice treated with melF10/A$^b$/B7.1 cells plus IL-12. Therapy with IL-12 alone also resulted in reduced lung metastases, as did therapy with melF10/A$^b$ cells plus IL-12 or therapy with melF10/B7.1 cells plus IL-12. Therapy with transfectants alone did not have a measurable effect. Treatment of mice carrying one week established melF10 lung metastases with melF10 tumor cells expressing syngeneic MHC class II plus CD80 plus systemic injection of IL-12, therefore, results in significant reduction in metastatic disease and the greatest reduction is seen when IL-12 is combined with the double

transfectant.

**Mice carrying established 4T1 mammary carcinoma metastases have reductions in metastatic disease following treatment with IL-12 plus 4T1 transfectants or wild type tumor.** To determine if the therapy effect seen in Table 1 and Figure 2 is applicable to additional tumors mice with established, spontaneous 4T1 mammary carcinoma metastases were also treated. Syngeneic BALB/c female mice were inoculated in the mammary fat pad with 7 X $10^3$ 4T1 tumor cells and tumors allowed to develop for 3 weeks at which time primary tumors ranged from 1-8 mm in diameter. Previous experiments ascertained that at this time and size of primary tumor, extensive metastatic disease is well established in the lungs, and that the number of metastatic cells in the lungs is roughly proportional to the size of the primary tumor (12). Therapy was started on day 21 after initial tumor challenge and consisted of 1 injection per week of $10^6$ irradiated tumor cells/transfectants (4T1, 4T1/$A^d$, 4T1/B7.1, or 4T1/$A^d$ + 4T1/B7.1) plus 3 injections/week of 1 µg IL-12/mouse. Mice were sacrificed after 3 weeks of therapy (day 42 after initial 4T1 inoculation), and the number of metastatic cells in the lungs measured by the clonogenic assay (12).

As shown in Figure 3, therapy with 4T1/$A^d$ plus 4T1/B7.1 transfectants reduces the number of metastases in the lungs, however, 67% of the mice (6/9) still have >22,000 metastatic tumor cells in their lungs (panel C). Treatment with IL-12 alone causes some reduction in the number of clonogenic metastatic cells relative to control 4T1 treated mice (panel B vs. panel A), however, 44% of mice (8/18) still have >10,000 metastatic cells in their lungs (panel B). In contrast, treatment with IL-12 plus any transfectant or IL-12 plus wild type 4T1 results in dramatic reduction in the number of

lung metastases in individual mice and a reduction in the number of mice with detectable metastatic cells (panels d-g) vs. mice treated with 4T1 cells alone (panel a). Pooling all of the groups treated with cells plus IL-12 (panels d-g) shows that only 21% of mice (11/53) have >10,000 metastastic tumor cells in the lungs following treatment (Tukey's $p < 0.05$). Maximum reduction of established, spontaneous 4T1 lung metastases, therefore, occurs following combination therapy of IL-12 plus irradiated tumor cells, however, wild type tumor cells are as effective as transfectants in the combined therapy.

**Combination IL-12 and cellular therapy affects growth of smaller primary, solid tumors.** Previous studies have demonstrated that IL-12 therapy mediates tumor regression of relatively small, primary, solid tumors. To confirm these observations and to determine if the presence of metastatic disease complicates IL-12 therapy, we have assessed 4T1 primary tumor growth in mice with metastases and treated with IL-12 and/or cellular therapy. Female BALB/c mice were inoculated in the abdominal mammary fat pad with $7 \times 10^3$ wild type 4T1 cells. As in figure 3, IL-12 and/or cellular therapy was initiated on day 21 and continued for 3 weeks. Primary tumor diameters were measured at the beginning of therapy and 2-3 times per week thereafter. At the end of 3 weeks of therapy, mice were categorized as follows: Regressor mice (R) showed a marked reduction in primary tumor diameter. Non-responder mice (NR) showed continued growth of primary tumor. Partial-responder mice (PR) showed no change in tumor size. To determine if primary tumor burden at the start of therapy affects therapeutic outcome, mice were analyzed according to tumor diameter at the start of treatment. As shown in Table 2, regardless of the therapy (IL-12 alone or IL-12 plus cells), all mice with small tumors at the start of therapy (< 2 mm in diameter) were

regressor mice. In contrast, mice with larger tumors at the start of therapy responded heterogeneously. For mice with 2-3.9 mm tumors, IL-12 plus 4T1/$A^d$/B7.1 cells produced the largest number of regressor mice, however all therapies (IL-12 alone or IL-12 plus any cells) produced approximately equally responses if regressor and partial-responder mice are pooled. In contrast, there were fewer regressor and partial regressor mice if therapy was started when tumors were 4-5.9 mm in diameter, and responses to IL-12 alone or IL-12 plus cells did not differ. For mice with starting tumors of > 6mm in diameter there were no regressors or partial responders. In agreement with studies by other investigators, IL-12 therapy alone, therefore, mediates regression or partial regression of tumors of <6 mm in diameter in a subset of treated mice. However, combination therapy of IL-12 plus tumor cells causes more complete responses if starting tumors are between 2-4 mm in diameter.

**Immunotherapy effect is not exclusively dependent on $CD4^+$, $CD8^+$, or NK cells.** The concept of combining a cell-based immunization therapy with IL-12 was based on the hypothesis that IL-12 facilitates the development of $T_{h1}$-type $CD4^+$ T helper lymphocytes (31) that are activated by immunization with the MHC class $II^+B7.1^+$ tumor cells. These $T_{h1}$ cells would, in turn, provide "help" to tumor-specific $CD8^+$ T lymphocytes and improved anti-tumor immunity would result. To test this hypothesis and to determine the involvement of T cells, mice undergoing immunotherapy were depleted for $CD4^+$ and $CD8^+$ T cells. Depletions were performed as described in Materials and Methods, and were initiated prior to the first cycle of immunotherapy. These experiments were completed in both the melF10 and 4T1 systems, and the immunotherapy protocols for each tumor were identical to those used in the experiments

#1 and #2 of Table 1 (B16melF10 tumor) and figure 3 (4T1 tumor). As shown in Table 1, experiment #3, and Figure 3, panels H-J, depletion of $CD4^+$ or $CD8^+$T cells does not diminish the therapeutic effect for the 4T1 tumor, while depletion of $CD8^+$ T cells, but not $CD4^+$ T cells, somewhat reduces the therapeutic effect for the B16melF10 tumor. These observations indicate that although $CD8^+$ T cells may have some role in B16melF10 growth regulation, cells other than conventional T cells are also responsible for reduction of metastases.

In addition to its role in $T_{h1}$ development, IL-12 also stimulates NK cell function (32). Mice undergoing immunotherapy, therefore, were also tested for NK cell levels and NK activity. Splenocytes of C57BL/6 mice and BALB/c mice carrying established melF10 and 4T1 metastases, respectively, and having at least 2 weeks of immunotherapy were tested for NK1.1(C57BL/6) or pan NK (BALB/c), $CD4^+$, $CD8^+$, $CD3^+$, and $B220^+$ expression. The percentages of $CD4^+$, $CD8^+$, and $B220^+$ splenocytes did not change following therapy with cells and/or IL-12 (data not shown). In contrast, as shown in Table 3, the percentage of $NK1.1^+$ splenocytes in C57BL/6 and pan $NK^+$ splenocytes in BALB/c mice was higher in all treated mice relative to untreated control mice. In mice carrying the melF10 tumor many of the $NK^+$ splenocytes also co-expressed CD3, as shown by double staining with NK1.1 and CD3 mAbs. The increase in NK phenotype was only seen in mice receiving therapy, because untreated mice carrying tumor did not have elevated levels of $NK^+$ splenocytes. Therapy of tumor-bearing mice with IL-12 and/or tumor cells, therefore increases NK cell levels in the hosts, suggesting the possibility that NK cells are involved in the therapeutic effect.

To determine if the increase in splenic NK levels resulted in an increase in functional NK cell activity, splenocytes from melF10 therapy mice and naive mice were

tested as effector cells in NK assays with P815 and YAC-1 target cells. Tumor-free, non-therapy C57BL/6 mice given poly IC 24 hours before assay to stimulate NK activity served as positive controls for measuring NK functional activity. As shown in Table 3, therapy mice did not have increased NK functional activity compared to untreated or tumor-free mice, while poly IC-boosted mice showed NK activity of 23-38% killing at E:T ratios of 50:1 splenocytes:YAC-1 targets. Cytotoxicity against P815 cells, a non-susceptible NK target, was uniformly <5%. Mice treated with IL-12 and/or cell-based immunotherapies, therefore, have increased phenotypic levels of $NK^+$ splenocytes, but these splenocytes do not display increased cytotoxic function as measured in vitro.

To determine if NK cells are responsible for the anti-metastatic effects of the cell-based therapy on 4T1 tumor growth, therapy mice were depleted for NK cells using anti-asialo-GM1 polyclonal antibodies prior to initiation of therapy. As shown in Figure 3, panel K, therapy mice depleted for NK cells have more metastatic tumor cells in their lungs than non-NK depleted mice, however, they do not have levels of metastatic cells as high as untreated mice. NK cells, therefore, may mediate some of the therapy effect, however, other effector mechanisms are probably also involved.

Although neither T nor NK cells are exclusively responsible for the therapeutic effect, these cell populations may be functioning cooperatively to diminish growth of metastatic cells. To test this hypothesis, beige/nude/XID mutant mice, which are deficient for NK and T cells, were given 4T1 tumor in the mammary fat pad on day 1, and therapy with 4T1 cells alone, IL-12 alone, or 4T1 cells plus IL-12 initiated on day 21. As shown in figure 4, therapy with IL-12 plus cells results in fewer metastatic cells in the lungs as compared to therapy with 4T1 alone, however, the reduction is not as dramatic as that seen in fully immunocompetent mice (see figure 3 for therapy of

immunocompetent mice). Beige/nude/XID mice, therefore, respond to the therapy (t-test, $p < 0.003$), however, the response is not as great as that seen in immunocompetent mice, suggesting that NK and T cells are partially responsible for the therapy effect.

**4T1 and MelF10 tumor cells are induced by IFN-γ to express the chemokine Mig.** Recent studies indicate that IL-12 and its downstream mediator IFN-γ may regulate tumor growth by stimulating anti-angiogenic chemokines including Monokine Induced by IFN-γ (Mig) and IFN-γ-Inducible Protein 10 (IP-10) (1, 2, 33). To determine if our combination therapy involves Mig and/or IP-10, RNA was prepared from the lungs of tumor-bearing therapy mice, reverse-transcribed, and PCR amplified using mig-specific and IP-10-specific PCR primers. Although IP-10 was not expressed in the lungs (data not shown), as shown in Figure 5, Mig mRNA is present in the lungs of 4T1 tumor-bearing mice receiving immunotherapy as early as 4 hours after initiation of therapy, and Mig expression is maintained throughout the 21 day duration of the therapy (figure 5, panel C), while control untreated, tumor-free mice do not express Mig in their lungs (panel A).

To determine if the tumor cells synthesize Mig and therefore produce the Mig detected in the lungs, 4T1 and MelF10 cells were cultured with IFN-γ for 2 hours in vitro and Mig and β-actin expression analyzed by reverse PCR. As shown in figure 6, IFN-γ induces expression of Mig in both 4T1 and MelF10 tumor cells.

## DISCUSSION

Several studies have reported a synergistic therapy effect when CD80-transfected tumor cells are combined with IL-12 and used as a vaccine to protect mice against a subsequent or simultaneous challenge of tumor (20, 21, 34, 35). Other studies have shown therapeutic efficacy of CD80-transfected tumor cells combined with IL-12 in the treatment of very short-term established experimental lung metastases (16, 36), or longer term established primary, solid tumors (17). With the exception of one study which reported that IL-12 up-regulated B7 expression on host APC (37), all of these studies demonstated more effective immunity when IL-12 and CD80 were combined.

Although all of these studies showed potent anti-tumor activity and reductions in tumor growth, only one of the reports examined spontaneous metastatic disease (38), and none of the reported studies addressed longer term experimental or spontaneous metastatic disease. In addition, the previously reported studies have focused exclusively on activation of $CD8^+$ T cells, although CD80 plus IL-12 are potent activators of $CD4^+$ T cells (13, 14). Since much metastatic disease is poorly responsive to conventional treatments and immunotherapy is a potential alternative treatment, we have incorporated the CD80 plus IL-12 combined therapy with our previously developed approach targeting the activation of $CD4^+$ T cells, and tested the combined approach in two mouse tumor systems in which metastatic lesions are longer established and/or arise spontaneously.

In both the B16melF10 and 4T1 tumor systems the combination IL-12 plus cell

based vaccine therapy produces significant reduction in metastases. The role of transfected tumor cells, however, differs between the two tumors. For the B16melF10 tumor, treatment with IL-12 plus MHC class II, CD80-transfected tumor cells provides the greatest reduction in metastases, while for the 4T1 tumor, treatment with IL-12 plus any transfectant or wild type tumor gives equivalent protection. As shown in the summary Table 4 for the 4T1 tumor, therapy with IL-12 alone is just as effective as therapy with cells plus IL-12 if the primary tumor is <2 mm in diameter at the start of therapy. In contrast, if the primary tumor is >2mm in diameter at the start of therapy, then therapy with cells plus IL-12 is considerably more effective than IL-12 therapy alone.

The combination therapy of cells plus IL-12 is also more effective than IL-12 therapy alone for the treatment of primary tumors. As shown in the summary Table 4, the combination therapy produces more responder mice than IL-12 alone regardless of the size of the primary tumor at the initiation of therapy.

Since IL-12 is known to stimulate $T_{h1}$ lymphocyte activity (13, 14, 31), our expectation was that $CD4^+$ T cells would be required for the immunotherapy effect. Contrary to this expectation, $CD4^+$ T cells were not obviously required in either tumor system, and although $CD8^+$ T cells have some effect in the B16melF10 tumor, they are not obviously involved in the therapy of the 4T1 tumor. Because IL-12 also stimulates NK cell activity, it was also anticipated that increased NK activity may contribute to the therapeutic effect. Although splenocytes with an NK phenotype as measured by immunofluorescence were increased in therapy mice, there was no concomitant increase in NK functional activity and in vivo NK depletions did not impact immunotherapy, further suggesting that NK cells are not responsible for the therapy

effect. These results also do not support a recent report attributing IL-12-mediated tumor rejection to a novel population of MHC un-restricted effector cells, $V_{\alpha}14$ NKT cells (39). Further confounding the identification of the effector cells in the combination therapy is the observation that mice deficient for NK and T cells (CR:NIH-beige/nude/XID mice) remain at least partially responsive to the therapy, suggesting that effector cells other than T and NK are also involved in the therapy effect.

As recently suggested by several other studies, IL-12 stimulates IFN-γ production, which in turn stimulates expression of chemokines such as Mig that may either directly or indirectly affect tumor growth (1, 2, 33). Mig and/or IL-12 could be affecting tumor growth by at least three mechanisms: 1) Because Mig is a chemoattractant for T cells and NK cells (40) it may be facilitating migration of these effectors to the lungs where they mediate tumor cell destruction. 2) Because of their anti-angiogenic activity, Mig and IL-12 may be directly or indirectly limiting tumor-mediated angiogenesis (1, 2, 40-43). 3) Because IL-12 causes tumor necrosis, the lung metastases may become necrotic (44). Collectively, the antibody depletion, chemokine induction, and immunodeficient mouse experiments suggest that the combined IL-12 and cellular vaccine therapy induces a combination of effector cells and effector molecules, including T cells, NK cells, neutrophils, and chemokines that synergistically diminish growth of lung metastases.

Mice treated in these studies had extensive tumor burdens and metastatic disease, much more advanced than mice commonly used in experimental immunotherapy protocols. Although the precise mechanism and contributions of each effector cell type and/or factor responsible for the anti-therapeutic effect is unclear, the

combined use of tumor cells/transfectants plus IL-12 produces a more potent anti-tumor effect than either IL-12 or tumor cells/transfectants alone and the combined use of these reagents in clinical immunotherapy protocols should be considered.

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## References


1. Coughlin, C., Salhany, K., Gee, M., LaTemple, C., Kotenko, S., Ma, X., Gri, G., Wysocka, M., Kim, J., Liu, L., Liao, f., Farber, J., Pestka, S., Trinchieri, G., and Lee, W. Tumor cell responses to IFN-gamma affect tumorigenicity and response to IL-12 therapy and antiangiogenesis., Immunity. *9:* 25-34, 1998.

2. Kanegane, C., Sgadari, C., Kanegane, H., Teruya-Feldstein, J., Yao, L., Gupta, G., Farber, J., Liao, L., and Tosato, G. Contribution of the CXC chemokines IP-10 and Mig to the antitumor effects of IL-12., J. Leukoc. Biol. *64:* 384-1448, 1998.

3. Brunda, M., Luistro, L., Warrier, L., Wright, R., Hubbard, B., Murphy, M., Wolf, S., and Gately, M. Anti-tumor and anti-metastatic activity of IL-12 against murine tumors., J. Exp. Med. *178:* 1223-1230, 1993.

4. Tahara, H., Zeh, H., Storkus, W., Pappo, I., Watkins, C., Gubler, U., Wolf, S., Robbins, P., and Lotze, M. Fibroblasts genetically engineered to Secrete interleukin-12 can suppress tumor growth and induce antitumor immunity to a murine melanoma in vivo., Canc. Res. *54:* 182-189, 1994.

5. Tahara, H., Zitvogel, L., Storkus, W., Zeh, H., McKinney, T., Schreiber, R., Gubler, U., P., R., and Lotze, M. Effective eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector., J. Immunol. *154:* 6466-6474, 1995.

6. Townsend, S. E. and Allison, J. P. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells, Science. *259:* 368-370, 1993.

7. Chen, L., Ashe, S., Brady, W. A., Hellstrom, I., Hellstrom, K. E., Ledbetter, J. A.,

McGowan, P., and Linsley, P. S. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4, Cell. *71:* 1093-1102, 1992.

8. Ostrand-Rosenberg, S., Thakur, A., and Clements, V. Rejection of mouse sarcoma cells after transfection of MHC class II genes, J. Immunol. *144:* 4068-4071, 1990.

9. Ostrand-Rosenberg, S. Tumor immunotherapy: The tumor cell as an antigen-presenting cell, Curr. Opin. Immunol. *6:* 722-727, 1994.

10. Baskar, S., Ostrand-Rosenberg, S., Nabavi, N., Nadler, L. M., Freeman, G. J., and Glimcher, L. H. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules, Proc. Natl. Acad. Sci. USA. *90:* 5687-5690, 1993.

11. Baskar, S., Glimcher, L., Nabavi, N., Jones, R. T., and Ostrand-Rosenberg, S. Major histocompatibility complex class $II^+B7\text{-}1^+$ tumor cells are potent vaccines for stimulating tumor rejection in tumor-bearing mice, J. Exp. Med. *181:* 619-629, 1995.

12. Pulaski, B. and Ostrand-Rosenberg, S. MHC class II and B7.1 immunotherapeutic cell-based vaccine reduces spontaneous mammary carcinoma metastases without affecting primary tumor growth., Canc. Research. *58:* 1486-1493, 1998.

13. Kubin, M., Kamoun, M., and Trinchieri, G. Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T

cells, J. Exp. Med. *180:* 211-222, 1994.

14. Murphy, E., Terres, G., Macatonia, E., Hsieh, C., Mattson, J., Lanier, L., Wysocka, M., Trinchieri, G., Murphy, K., and O'Garra, A. B7 and Interleukin 12 cooperate for proliferation and interferon gamma production by mouse T helper clones that are unresponsive to B7 costimulation, J. Exp. Med. *180:* 223-231, 1994.

15. Gajewski, T., Renauld, J., Van Pel, A., and Boon, T. Costimulation with B7-1, IL-6, and IL-12 is sufficient for primary generation of murine antitumor cytolytic T lymphocytes in vitro., J. Immunol. *154:* 5637-5648, 1995.

16. Coughlin, C., Wysocka, M., Kurzawa, H., Lee, W., Trinchieri, G., and Eck, S. B7-1 and interleukin 12 synergistically induce effective antitumor immunity., Cancer Res. *55:* 4980-4987, 1995.

17. Putzer, B., Hitt, M., Muller, W., Emtage, P., Gauldie, J., and Graham, F. Interluekin 12 and B7-1 costimulatory molecule expressed by an adenovirus vector act synergistically to facilitate tumor regression., Proc. Natl. Acad. Sci. USA. *94:* 10889-10894, 1997.

18. Colombo, M., Vagliani, M., Spreafico, F., Parenza, M., Chiodoni, C., Melani, C., and Stoppacciaro, G. Amount of Interleukin-12 available at the tumor site is critical for tumor regression., Canc. Research. *56:* 2531-2534, 1996.

19. Chen, L., Chen, D., Block, E., O'Donnell, M., Kufe, D., and Clinton, S. Eradication of murine bladder carcinoma by intratumor injection of a bicistronic adenoviral vector carrying cDNAs for the IL-12 heterodimer and its inhibition by the iL-12 p40

subunit homodimer., J. Immunol. *159:* 351-359, 1997.

20. Chong, H., Todryk, S., Hutchinson, G., Hart, I., and Vile, R. Tumor cell expression of B7 costimulatory molecules and interleukin-12 or granulocyte-macrophage colony-stimulating factor induces a local antitumour response and may generate systemic protective immunity, Gene Ther. *5:* 223-232, 1998.

21. Pizzoferrato, E., Chu, N., Hawley, T., Lieu, F., Barber, B., Hawley, R., Watts, T., and Berinstein, N. Enhanced immunogenicity of B cell lymphoma genetically engineered to express both B7-1 and interleukin-12., Hum. Gene Ther. *8:* 2217-2228, 1997.

22. Miller, F., Miller, B., and Heppner, G. Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability., Invasion Metastasis. *3:* 22-31, 1983.

23. Fidler, J. and Hart, I. Biological diversity in metastatic neoplasms: origins and implications, Science. *217:* 998-1003, 1982.

24. Ostrand-Rosenberg, S., Baskar, S., Patterson, N., and Clements, V. Expression of MHC class II and B7-1 and B7-2 costimulatory molecules accompanies tumor rejection and reduces the metastatic potential of tumor cells., Tissue Antigens. *47:* 414-421, 1996.

25. Janeway, C., Conrad, P., Lerner, E., Babich, J., Wettstein, P., and Murphy, D. Monoclonal antibodies specific for Ia glycoproteins raised by immunization with activatedT cells: possible role of t cell-bound Ia antigens as targets of immunoregulatory T cells., J. Immunol. *132:* 662-667, 1984.

26. Kappler, J., Skidmore, B., White, J., and Marrack, P. Antigen-inducible, H-2-restricted, IL-2 producing T cell hybridomas. Lack of independent antigen and H-2 recognition., J. Exp. Med. *153:* 1198-1214, 1981.

27. Ozato, K. and Sachs, D. Monoclonal antibodies to mouse MHC antigens., J. Immunol. *126:* 317-321, 1981.

28. Wilde, D., Marrack, P., Kappler, J., Dialynis, D., and Fitch, F. Evidence implicating L3T4 in class II MHC antigen reactivity: monoclonal antibody GK1.5 blocks class II MHC antigen-specific proliferation, release of lymphokines, and binding by cloned murine helper T lymphocyte lines., J. Immunol. *131:* 2178-2183, 1983.

29. Sarmiento, M., Glasebrook, A., and Fitch, F. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt2 antigen block T cell mediated cytolysis in the absence of complement., J. Immunol. *125:* 2665-2672, 1980.

30. Nishimura, M. I., Stroynowski, I., Hood, L., and Ostrand-Rosenberg, S. H-2K$^b$ antigen expression has no effect on natural killer susceptibility and tumorigenicity of a murine hepatoma, J. Immunol. *141:* 4403-4409, 1988.

31. Hsieh, C., Macatonia, S., Tripp, C., Wolf, S., O'Garra, A., and Murphy, K. Development of $T_H1$ CD4$^+$ T cells through IL-12 produced by Listeria-induced macrophages., Science. *260:* 547-549, 1993.

32. Brunda, M. Interleukin-12, J. Leuk. Biol. *55:* 280-288, 1994.

33. Tannenbaum, C., Tubbs, R., Armstrong, D., Finke, J., Bukowski, R., and Hamilton, T. The CXC chemokines IP-10 and Mig are necessary for IL-12-

mediated regression of the mouse RENCA tumor, J. Immunol. *161:* 927-932, 1998.

34. Chen, P., Geer, D., Podack, E., and Ksander, B. Tumor cells transfected with B7-1 and interleukin-12 cDNA induce protective immunity., Ann. NY Acad. Sci. *795:* 325-327, 1996.

35. Zitvogel, L., Robbins, P., Storkus, W., Clarke, M., Maeurer, M., Campbell, R., Davis, C., Tahara, H., Schreiber, R., and Lotze, M. Interleukin-12 and B7.1 co-stimulation cooperate in the induction of effective antitumor immunity and therapy of established tumors, Eur. J. Immunol. *26:* 1335-1341, 1996.

36. Rao, J., Chamberlain, R., Bronte, V., Carroll, M., Irvine, K., Moss, B., Rosenberg, S., and Restifo, N. IL-12 is an effective adjuvant to recombinant vaccinia virus-based tumor vaccines: enhancement by simultaneous B7-1 expression., J. Immunol. *156:* 3357-3365, 1996.

37. Fallarino, F., Ashikari, A., Boon, T., and Gajewski, T. Antigen-specific regression of established tumors induced by active immunization with irradiated IL-12 but not B7-1 transfected tumor cells., Int. Immunol. 1259-1269, 1997.

38. Kato, K., Okumura, K., and Yagita, H. Immunoregulation by B7 and IL-12 gene transfer, Leukemia. *11, Supp. 3:* 572-576, 1997.

39. Cui, J., Shin, T., Kawano, T., Sato, H., Kondo, E., Toura, I., Kaneko, Y., Koseki, H., Kanno, M., and Taniguchi, M. Requirement for V alpha 14 NKT cells in IL-12-mediated rejection of tumors., Science. *278:* 1623-1626, 1997.

40. Farber, J. Mig and IP-10: CXC chemokines that target lymphocytes., J. Leukoc.

Biol. *61:* 246-257, 1997.

41. Cavallo, F., Di Carlo, E., Butera, M., Verrua, R., Colombo, M., Musiani, P., and Forni, G. Immune events associated with the cure of established tumors and spontaneous metastases by local and systemic interleukin 12., Canc. Res. *59:* 414-421, 1999.

42. Sgadari, C., Angiolillo, A., and Tosato, G. Inhibition of angiogenesis by interleukin-12 is mediated by the interferon-inducible protein 10., Blood. *87:* 3877-3882, 1996.

43. Yao, L., Sgadari, C., Furuke, K., Bloom, E., Teruya-Feldstein, J., and Tosato, G. Contribution of natural killer cells to inhibition of angiogenesis by interleukin-12., Blood. *93:* 1612-1621, 1999.

44. Sgadari, C., Farber, J., Angiolillo, A., Liao, f., Teruya-Feldstein, J., Burd, P., Yao, L., Gupta, G., Kanegane, C., and Tosato, G. Mig, the monokine induced by interferon-gamma, promotes tumor necrosis in vivo., Blood. *89:* 2635-2643, 1997.

**Table 1.** Mice carrying established i.v. B16melF10 melanoma metastatses have reduced lung metastases following treatment with melF10/A$^b$/B7.1 plus IL-12.

| Exp. # | Treatment | | | Mean Lung Weight (mg) |
|---|---|---|---|---|
| | Therapeutic Cells | IL-12 | T Cell Depletion | |
| 1 | --- | --- | --- | 995 |
| | --- | IL-12 | --- | 468[a] |
| | melF10/A$^b$/B7.1 | --- | --- | 990 |
| | melF10/A$^b$/B7.1 | IL-12 | --- | 337[a] |
| 2 | --- | --- | --- | 1025 |
| | --- | IL-12 | --- | 454[a] |
| | melF10/A$^b$ | IL-12 | --- | 337[a] |
| | melF10/B7.1 | IL-12 | --- | 409[a] |
| | melF10/A$^b$/B7.1 | IL-12 | --- | 288[a] |
| 3 | --- | --- | | 492[c] |
| | melF10 | IL-12 | --- | 313[b] |
| | melF10/A$^b$/B7.1 | IL-12 | --- | 277[b] |
| | melF10/A$^b$/B7.1 | IL-12 | CD4 depleted[e] | 250[b,d] |
| | melF10/A$^b$/B7.1 | IL-12 | CD8 depleted[f] | 325[b] |

[a] These groups are significantly different from their untreated control group ($p<0.005$)

[b] These groups are not significantly different from each other.

[c] Two mice in this group died of tumor before the experiment was concluded and their lung weights are not included in this value.

[d] This group is significantly different from the untreated control group ($p<0.05$).

[e] Mice were 90-100% depleted for $CD4^+$ T cells.

[f] Mice were 100% depleted for $CD8^+$ T cells.

Syngeneic C57BL/6J mice were challenged i.v. on day 1 with $10^5$ wild type B16melF10 tumor cells. Beginning 6 days after tumor inoculation, and continuing for the following 3 weeks, mice were inoculated i.p. once a week with therapeutic cells and 3 times per week i.p. with 1 µg IL-12. Surviving mice were sacrificed on day 26 after initial tumor inoculation and lung weights determined. Each groups contains 5 mice.

**Table 2:** IL-12 plus cell therapy mediates regression of small and medium sized primary tumors, while IL-12 alone therapy only affects small primary tumors.

| Therapeutic Cells | IL-12 | No. Responding Mice/Total Mice Treated<br>Tumor Diameter at Start of Therapy (mm) | | | |
|---|---|---|---|---|---|
| | | 0-1.99 | 2-3.99 | 4-5.99 | >6 |
| 4T1 | --- | 2/2 (NR) | 6/6 (NR) | 7/7 (NR) | 1/1 (NR) |
| 4T1/A$^d$ + 4T1/B7.1 | --- | ND | 2/2 (NR | 6/6 (NR) | 1/1 (NR) |
| --- | IL-12 | 2/2 (R) | 1/7 (R)<br>4/7 (PR)<br>2/7 (NR) | 2/8 (PR)<br>6/8 (NR) | 2/2 (NR) |
| 4T1/A$^d$ | IL-12 | 1/1 (R) | 3/8 (R)<br>4/8 (PR)<br>1/8 (NR) | 2/2 (NR) | 1/1 (NR) |
| 4T1/B7.1 | IL-12 | 2/2 (R) | 1/3 (R)<br>1/3 (PR)<br>1/3 (NR) | 2/5 (R)<br>3/5 (PR) | ND |
| 4T1/A$^d$ + 4T1/B7.1 | IL-12 | 2/2 (R) | 4/6 (R)<br>1/6 (PR)<br>1/6 (NR) | 2/6 (PR)<br>4/6 (NR) | 1/1 (NR) |

ND, not determined

R, "responder," primary tumor diameter regressed during therapy

PR, "partial responder," primary tumor diameter remained the same during therapy

NR, "non-responder," primary tumor diameter continued to grow during therapy

**Table 3**. Mice carrying established melF10 or 4T1 metastases and treated with IL-12 and/or cell transfectants have increased levels of splenic NK , $CD80^+$, $B7.2^+$, and $Mac\text{-}1^+$ splenocytes.

| Primary Tumor | Therapy | | Percent Cells in Spleen[c] | | % Splenic NK Activity Against YAC-1 at 50:1 E:T ratio[f] |
|---|---|---|---|---|---|
| | Cells | IL-12 | $NK1.1^+$ or pan $NK^+$ | $NK1.1^+$ + $CD3^+$ | |
| none | --- | --- | 4 ± 0 | 2 ± 0 | ND |
| MelF10[a] | --- | --- | 6 ± 5 | 5 ± 3 | 5 |
| | --- | IL-12 | 25 ± 8[d] | 17 ± 6[d] | 2 |
| | MelF10 | --- | 10 ± 0[d] | 6 ± 1[d] | 8 |
| | MelF10 | IL-12 | 18 ± 8[d] | 14 ± 1[d] | ND |
| | MelF10/A[b]/B7.1 | IL-12 | 27 ± 6[d] | 21 ± 4[d] | 2 |
| none | --- | --- | 9 ± 0 | ND | ND |
| 4T1[b] | 4T1 | --- | 13 ± 2[e] | ND | ND |
| | --- | IL-12 | 14 ± 2[e] | ND | ND |
| | 4T1 | IL-12 | 13 ± 0[e] | ND | ND |

[a] MelF10 tumors were grown in C57BL/6 mice.

[b] 4T1 tumors were grown in BALB/c mice.

[c] Percent positive cells is the average number of cells for 2-8 mice per group in the 4T1 experiments and 2-9 mice per group in the melF10 experiments.

[d] These groups are significantly different from their untreated control group ($p<0.01$).

[e] These groups are significantly different from their untreated control group ($p<0.02$).

[f] Splenocytes from tumor-free, untreated mice given poly IC 24 hours prior to assay had NK cytotoxicity levels of 23-38% at 50:1 effector: YAC-1 ratios. Percent cytotoxicity against P815 cells for all effectors was <5%.

**Table 4:** Summary of primary and metastatic 4T1 tumor growth in BALB/c mice treated with IL-12 plus cell-based vaccine.

| Therapy | Percent Responder Plus Partial Responder Mice | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Primary Tumor Diameter at Start of Therapy (mm) | | | | | | | |
| | 0-1.99 | | 2-3.99 | | 4-5.99 | | ≥ 6 | |
| | 1° tumor[a] | mets[b] | 1° tumor | mets | 1° tumor | mets | 1° tumor | mets |
| 4T1 cells | 0 | 100 | 0 | 50 | 0 | 0 | 0 | 0 |
| IL-12 | 100 | 100 | 71 | 83 | 25 | 38 | 0 | 0 |
| cells + IL-12[c] | 100 | 100 | 82 | 100 | 44 | 52 | 0 | 66 |

a Mice are classified as "responders," "partial responders," or "non-responders." Diameter of primary tumor decreased in "responder" mice; diameter of primary tumor remained unchanged in "partial responder" mice; diameter of primary tumor increased in "non-responder" mice.

b Mice are classified as either "responders" or "non-responders." Responder mice have ≤10,000 clonogenic metastatic tumor cells in their lungs; non-responder mice have > 10,000 metastatic cells.

c pooled therapy groups using 4T1/A$^d$, 4T1/B7.1, or 4T1 tumor cells + IL-12

**Figure Captions:**

**Figure 1:** Flow cytometry profiles of melF10 and melF10 transfectants stained for MHC class I (H-2K$^b$ and H-2D$^b$), MHC class II (I-A$^b$), and B7.1 (CD80) molecules. Dotted lines are staining by fluorescent conjugate alone; solid lines are staining by specific antibody plus fluorescent conjugate.

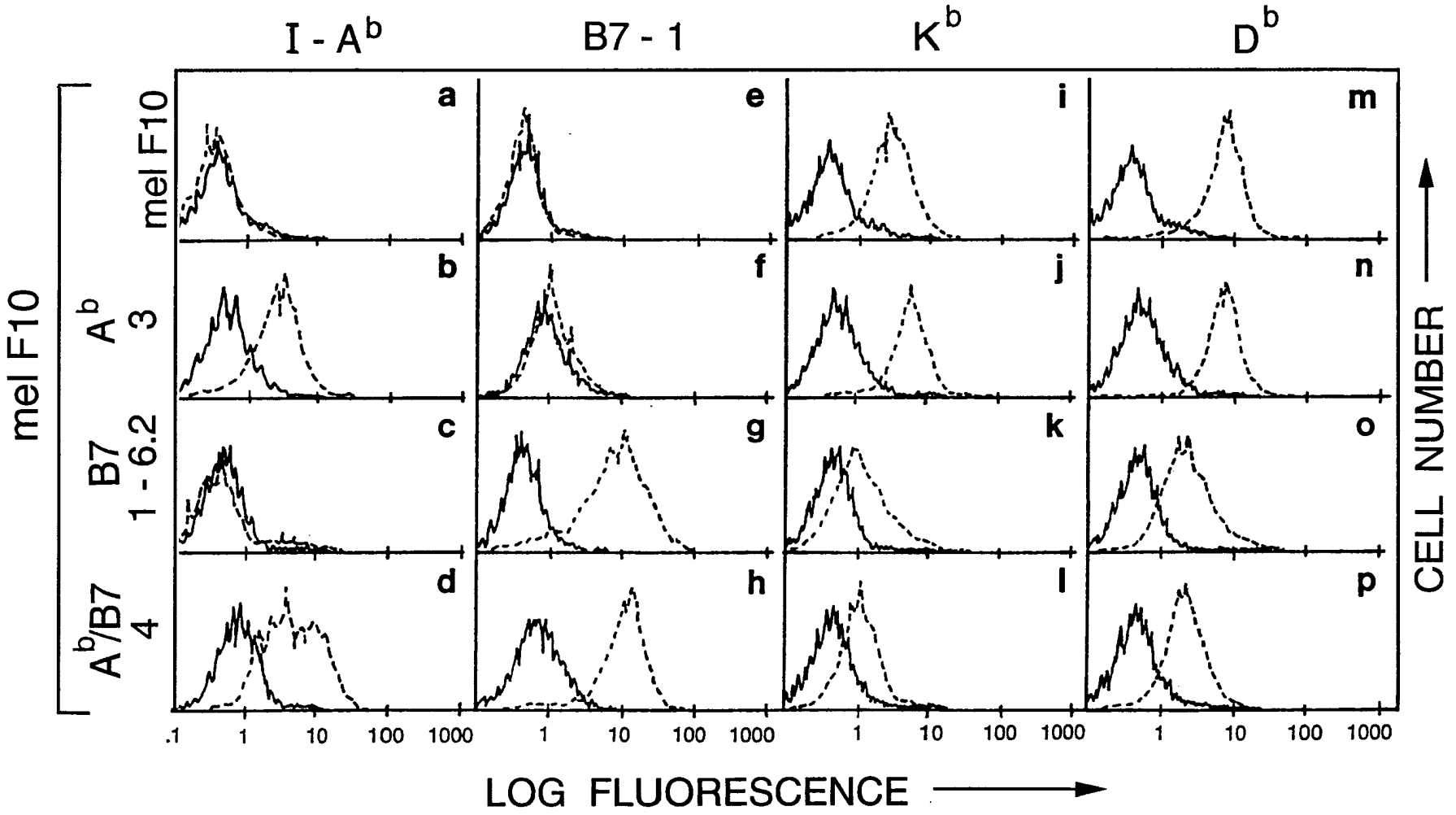
**Figure 2:** Metastatic tumor cells in the lungs of C57BL/6 mice treated with cell and/or IL-12 therapies. Mice were inoculated i.v. on day 0 with wild type melF10 tumor cells and therapy started on day 6. Therapy for each group is indicated. Following 3 weeks of therapy lungs were excised.. Each set of lungs is from an individual mouse.

**Figure 3:** Number of metastatic cells in BALB/c mice carrying established wild type 4T1 metastases and treated with cell and/or IL-12 therapies. Therapy for each group is indicated within each panel. Mice were inoculated with wild type tumor on day 0 and therapy started on day 21. Each symbol represents an individual mouse. Using Tukey's Honestly Significant difference test, the data of panels (A and B, cells alone therapy) are significantly different ($p < 0.05$) from the data of panels (D, E, F, and G, cells plus IL-12 therapy).

**Figure 4:** Number of metastatic cells in CR:NIH-beige/nude/XID mice carrying established wild type 4T1 metastases and treated with cell and/or IL-12 therapy. Mice were inoculated with wild type tumor on day 0 and therapy started on day 21. Each symbol represents an individual mouse. Using the two-tailed t-test, the data of panel A is significantly different from the data of panels B plus C ($p < 0.003$).

**Figure 5:** Mig mRNA is expressed in the lungs of BALB/c mice carrying 4T1 tumors. BALB/c mice were inoculated in the mammary fat pad with 7 X $10^3$ 4T1 cells on day 0 and therapy started on day 21. Lungs were removed 4 h, 7 days, and 21 days after therapy was started and lung RNA was isolated, reverse transcribed, and PCR amplified using Mig-specific or β-actin-specific primers for the indicated number of cycles.

**Figure 6:** 4T1 and B16melF10 tumors are induced in vitro by IFN-γ to express Mig. RNA from 4T1 and melF10 tumor cells cocultured with/without IFN-γ for 2 h was reverse transcribed and amplified using Mig-specific or β-actin-specific primers.


I - A^b
B7 - 1
K^b
D^b
mel F10
A^b 3
B7 1 - 6.2
A^b/B7 4
mel F10
a
b
c
d
e
f
g
h
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n
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.1
1
10
100
1000
CELL NUMBER
LOG FLUORESCENCE

Figure 1

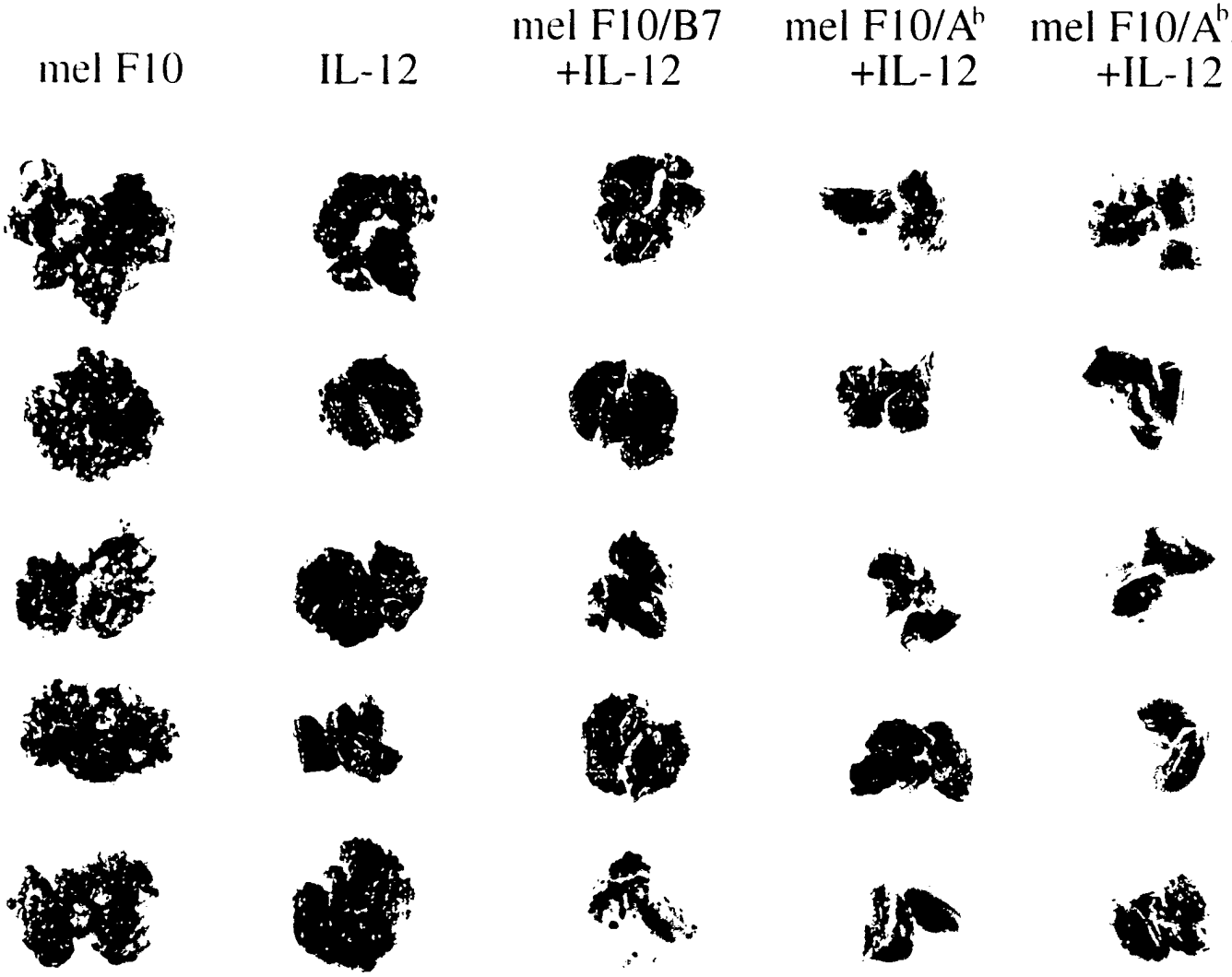


Figure 2

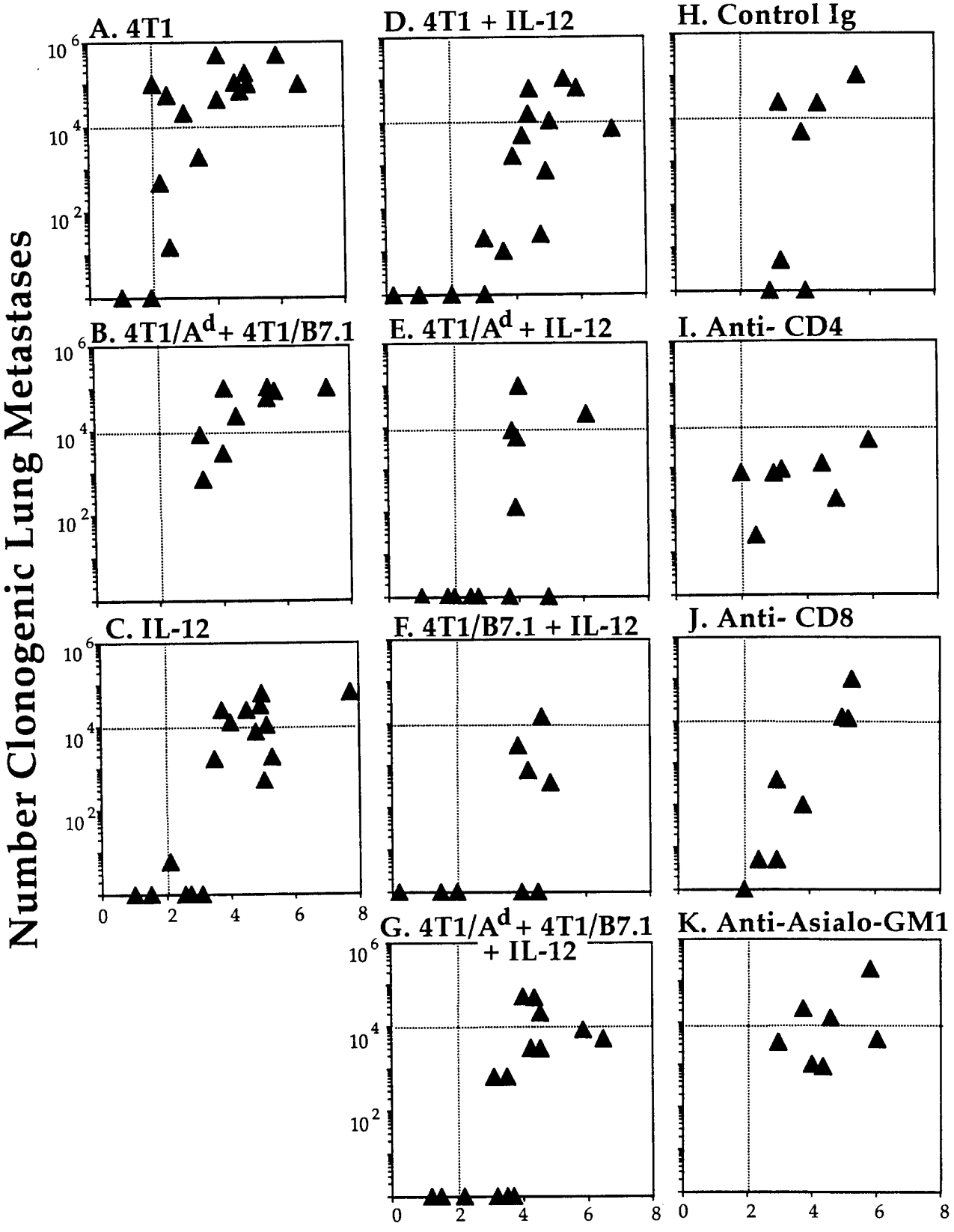
A. 4T1
D. 4T1 + IL-12
H. Control Ig
B. 4T1/A$^d$+ 4T1/B7.1
E. 4T1/A$^d$ + IL-12
I. Anti- CD4
C. IL-12
F. 4T1/B7.1 + IL-12
J. Anti- CD8
G. 4T1/A$^d$+ 4T1/B7.1 + IL-12
K. Anti-Asialo-GM1
Number Clonogenic Lung Metastases
Tumor Diameter (mm) at Start of Treatment

Figure 3

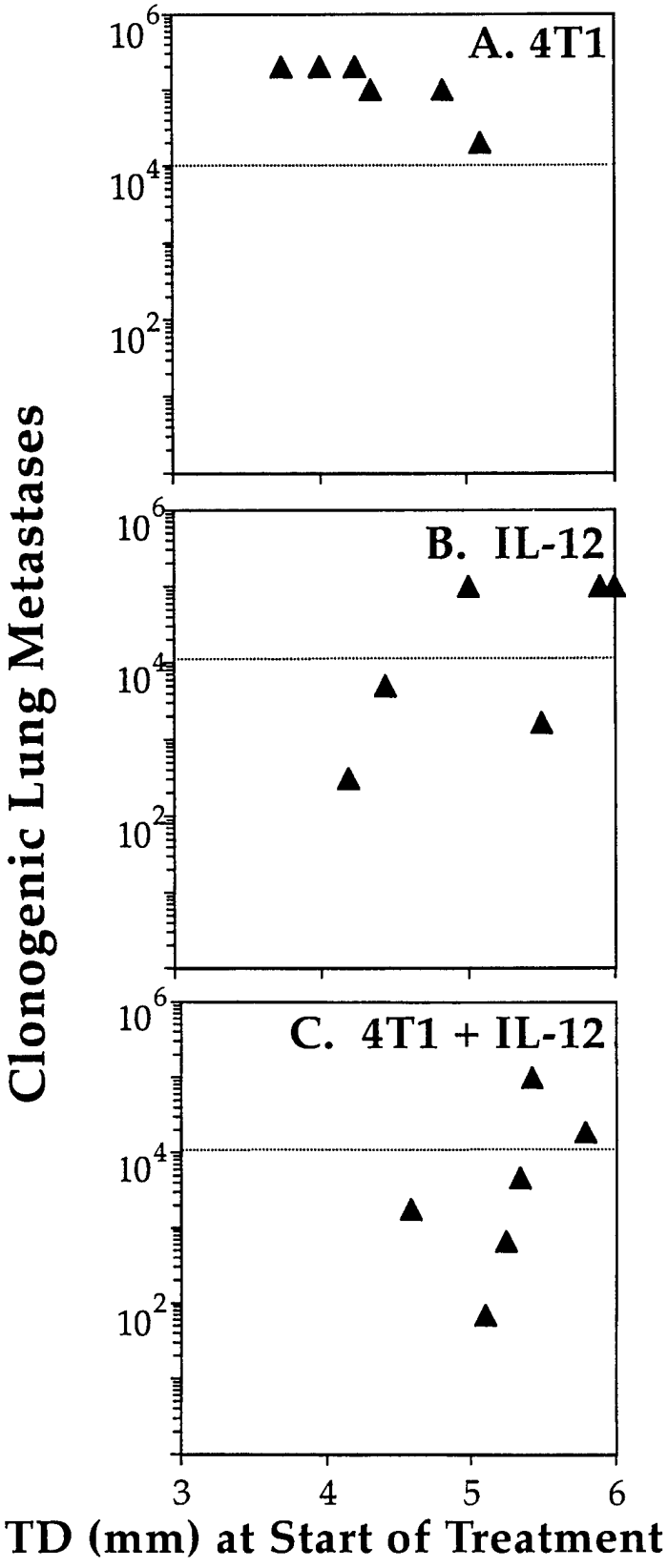
A. 4T1
B. IL-12
C. 4T1 + IL-12
Clonogenic Lung Metastases
$10^6$
$10^4$
$10^2$
3
4
5
6
TD (mm) at Start of Treatment

Figure 4

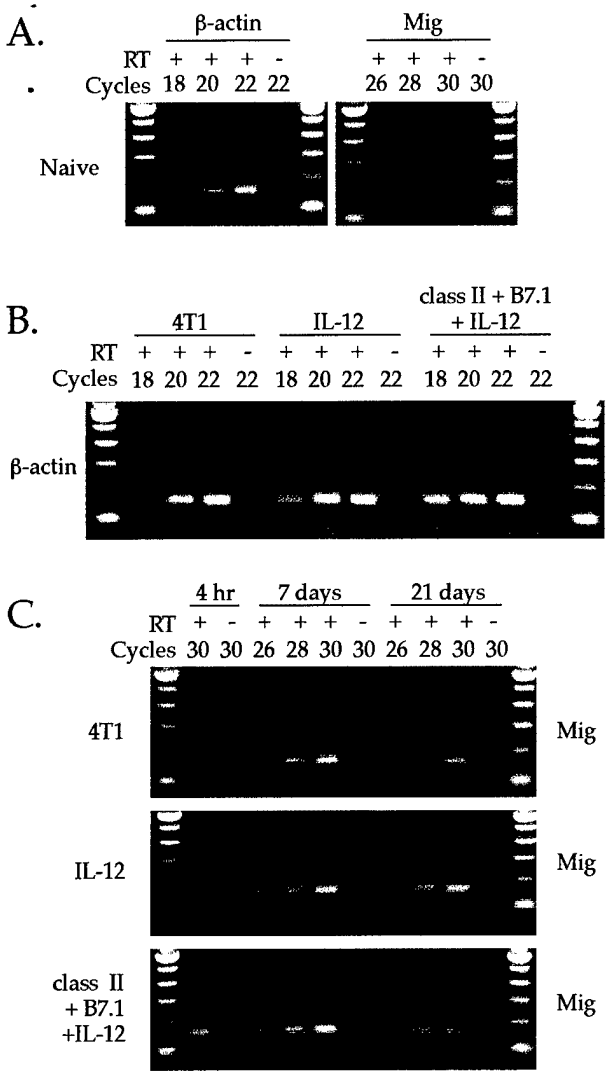
A.
β-actin
Mig
RT + + + - + + + -
Cycles 18 20 22 22 26 28 30 30
Naive
B.
4T1
IL-12
class II + B7.1 + IL-12
RT + + + - + + + - + + + -
Cycles 18 20 22 22 18 20 22 22 18 20 22 22
β-actin
C.
4 hr
7 days
21 days
RT + - + + + - + + + -
Cycles 30 30 26 28 30 30 26 28 30 30
4T1
Mig
IL-12
Mig
class II + B7.1 +IL-12
Mig

Figure 5

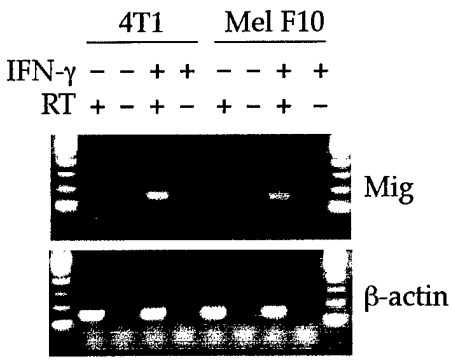


Figure 6

*Brief Definitive Report*

# Synergy of SEB Superantigen, MHC Class II, and CD80 in Immunotherapy of Advanced Spontaneous Metastatic Breast Cancer

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Running Title: spontaneous metastases reduction by SEB, class II, and B7.1

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Key Words: immunotherapy, SEB superantigen, MHC class II, B7.1 co-stimulation, spontaneous mammary carcinoma metastases

## ABSTRACT

No significant improvements in the treatment of metastatic breast cancer have been developed in the last 20 years and the prognosis for women with this disease remains poor. Progress in understanding the immune response, however, has led to renewed enthusiasm for immune-based anti-cancer therapies. In previous reports, we demonstrated that tumor cell-based vaccines expressing MHC class II and B7.1 (CD80) molecules reduced experimental (i.v.-induced) and established spontaneous metastatic disease, by activating tumor-specific $CD4^+$ T-lymphocytes. We now demonstrate, using the mouse 4T1 mammary carcinoma, that a vaccine combining MHC class II and B7.1 molecules with SEB superantigen in two distinct immunotherapeutic regimens produces an even greater reduction in spontaneous metastatic disease and significant extension of mean survival time. The therapeutic effect is particularly noteworthy because: 1) spontaneous metastatic cancer by 4T1 progresses similarly in comparison to human metastatic mammary cancer, 2) our post-operative model demonstrates that early metastatic lesions are primarily responsible for morbidity, and 3) the disseminated metastatic disease is extensive prior to the initiation of immunotherapy in both regimens.

## INTRODUCTION

As a result of recent discoveries/advances in immunology and molecular cloning, many novel immunotherapeutic strategies for the treatment of cancer are being developed (1-3). Most of these strategies are focused on eliminating primary tumors, which are frequently successfully treated by conventional methods, such as surgery. In contrast, very few immunotherapeutic approaches are targeting disseminated metastatic disease, for which there are frequently no effective conventional therapies. Development of therapies for the treatment of metastatic disease is complicated by the shortage of adequate animal models for spontaneously metastatic cancers. We recently described a novel cell-based vaccine for the therapy of metastatic disease and tested it using the mouse 4T1 mammary carcinoma. The 4T1 tumor shares many characteristics with its human counterpart, and hence is an excellent model system for testing experimental immunotherapies. In addition to its parallels with human disease, 4T1 cells are resistant to the drug 6-thioguanine (4), enabling one to precisely quantify the number of micro-metastatic cells in the lungs, LN, livers, blood, and brains of individual animals (5).

The cell-based vaccines consist of tumor cells transfected with syngeneic MHC class II and B7.1 co-stimulatory molecule genes and were designed to enhance activation of tumor-specific $CD4^+$ T lymphocytes via improved presentation of tumor-encoded class II-restricted epitopes. Although $CD8^+$ T lymphocytes have been traditionally the focus of immunotherapy approaches, accumulating results have demonstrated that $CD4^+$ T lymphocytes play a critical role in effective anti-tumor immunity (6-9). While our vaccine showed significant reduction of established,

spontaneous metastatic tumor, the anti-tumor response was limited to small burdens of metastatic cells and did not completely eliminate metastases (5). We now report a second-generation cell-based vaccine that is significantly more effective than the original vaccine for the treatment of spontaneous 4T1 metastatic mammary cancer. The new vaccine incorporates a gene encoding the bacterial toxin, *Staphylococcal aureus* enterotoxin B (SEB)[1]. SEB is a superantigen (sAg) that when complexed with MHC class II molecules on APC is a potent polyclonal activator of $CD4^+$ T lymphocytes (10, 11). Although $CD4^+$ T cell activation by SEB is not antigen-specific, we reasoned that addition of SEB to the MHC class II/B7.1 vaccine will provide additional activation signals to the $CD4^+$ T cells that have been activated in an antigen-specific fashion by the MHC class $II^+$ $B7.1^+$ vaccinating cells.

## MATERIALS AND METHODS

**cDNA Expression Vectors.** The expression vectors, pHβ-Apr-1-neo containing MHC class II (I-A$\alpha^d$, I-A$\beta^d$) and mouse B7.1, have been described previously (5). The SEB gene (12) was supplied by Dr. S. Khan and subsequently subcloned into the *Sal I/Bam HI* site of the pHβ-Apr-1-neo expression vector. The final constructs contained only the sequence within the coding region for each cDNA and conferred resistance to G-418. The pZeoSV2 plasmid was purchased from Invitrogen (San Diego, CA).

**Animals, Cell Lines, and Transfectants.** Female BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and/or bred in the UMBC animal facility and were used at 8 wk of age. 4T1, a 6-thioguanine-resistant cell line derived from a BALB/c spontaneous mammary carcinoma, was kindly supplied by Dr. Fred R. Miller (5). Unmodified tumor cells were cultured in IMDM media (GIBCO BRL, Gaithersberg, MD) supplemented with 10% fetal bovine product (Hyclone, Logan, Utah) and 1x Antibiotic-Antimycotic (GIBCO BRL). Transfectants were made to express MHC class II and B7.1, or SEB by using lipofectin (GIBCO BRL) according to manufacturer's instructions. Cells were selected with 400 μg/ml G-418 (GIBCO BRL) or 200 μg/ml zeocin (Invitrogen), cloned by limiting dilution, stained for surface antigen expression, and analyzed by flow cytometry as described previously (5).

**SEB Assay.** Naive BALB/c spleen cells (5-10 x$10^5$) were cultured in serial dilutions of transfectants' supernatants or purified SEB (Sigma, St. Louis, MO) as indicated. To demonstrate specific SEB activity, a polyclonal rabbit antibody against SEB (Sigma, catalog # S-9008) was added to cultures as indicated. After 3 d in

culture, spleen cell proliferation was measured using MTT reagent as previously described (13).

**In Vivo Tumor Growth and Spontaneous Metastases.** Mice were challenged s.c. in the abdominal mammary gland with either parental or transfected 4T1 tumor cells as indicated. Primary tumor growth and spontaneous metastases were measured as previously described (5). Animals were sacrificed when the mean tumor diameter (TD) reached 14-16 mm or when the mice became moribund, according to UMBC IACUC guidelines.

**Surgery.** All surgical supplies and surgical equipment were purchased from Henry Schein Veterinary & Medical Supply Catalog (Melville, NY) and Roboz (Rockville, MD), respectively, unless indicated otherwise. Prior to surgery animals were weighed and anesthetized with i.p. injections (0.02 cc/g body wt) of 2.5% recrystalized avertin (2,2,2-tribromomethanol, Sigma). Once the animals were unconscious, the tumor-bearing abdominal area was prepared and sterilized for surgery by shaving with Oster Finisher Trimmer (catalog # 216-0567) followed by 2 washes each with diluted Nolvasan surgical scrub (chlorhexidine, catalog # 995-3226) and isopropanol. Tumors were resected with sterilized surgical instruments, removing the smallest amount of skin tissue as possible. Wounds were closed with either Nexaband liquid (catalog # 893-8109) or stainless steel 9mm wound clamps (catalog # RS-9262) with a Mikon autoclamp applier (catalog # RS-9260) as necessary. Wound clamps were removed 10 d after surgery with a Mikon autoclamp remover (catalog # RS-9268). Mice were monitored for survival and those that died from surgery (within 1-4 d post surgery, survival rate of 77-80%) were not included in the

experiment. All mice were autopsied at the time of death to confirm the presence of lung metastases as well as recurrence of the primary tumor.

**Statistical Analyses.** To determine the statistical significance of the data, the student's t-test for unequal variances (Microsoft Excel version 5.0) and Tukey's Honestly Significant Difference Test, at a p-value equal to 0.05, were performed as indicated.

## RESULTS

*Expression of SEB by 4T1 transfectants reduces tumorigenicity and metastatic potential.* To test the anti-tumor effects of SEB, we transfected the SEB gene into the mouse-derived BALB/c 4T1 mammary carcinoma (4). The 4T1 tumor was chosen because it is very poorly immunogenic, highly malignant, spontaneously metastatic, and shows a disease progression pattern similar to human mammary tumors (5). Transfectants were selected in G-418 and cloned by limiting dilution. SEB expression was tested by co-culturing supernatants from 10 transfectants with naive BALB/c spleen cells and monitoring lymphocyte proliferation. To determine relative amount of SEB secretion, splenocytes were also cultured with soluble SEB. As shown in Figure 1, supernatants from two independent clones (4T1/SEB-12 and 4T1/SEB-14) stimulated splenocyte proliferation as efficiently as soluble SEB at a concentration of 2 μg/ml. This activity was reduced upon addition of a polyclonal anti-SEB antibody, demonstrating that the spleen cell proliferation was due to SEB expression by the 4T1 transfectants. Supernatants from parental 4T1 cells as well as supernatants from 4T1 cells transfected with empty vector (4T1/neo) did not induce proliferative responses (Figure 1 and data not shown). Therefore, the 4T1/SEB transfectants secrete SEB, which induces splenocyte proliferation comparable to proliferation induced by soluble exogenously added SEB.

To test the immunogenicity and tumorigenicity of the SEB transfectants, syngeneic female BALB/c mice were challenged in the abdominal mammary gland with $5x10^3$ tumor cells and the challenged mice followed for primary tumor growth and metastasis formation. Because 4T1 is 6-thioguanine resistant, small numbers of

metastatic cells can be readily detected by explanting target organs and culturing the dissociated cells in media supplemented with 6-thioguanine. Figure 2 shows the number of clonogenic tumor cells in the lungs vs. tumor diameter (TD) at time of sacrifice (large panels), and the growth rate of the primary tumor (inset panels) for the 2 transfectants. Both transfectants show some reduction in tumorigenicity (Figures 2B-C, inset panels), although, only the 4T1/SEB-12 transfectant does not form primary tumors in any of the inoculated mice (Figure 2B, inset panel). In contrast, the metastatic potential of both SEB transfectants is markedly reduced relative to 4T1 cells. For example, 8/10 mice inoculated with SEB transfectants contained no metastatic cells in the lung (Figure 2B-C, large panels), while 5/5 mice inoculated with wild type 4T1 cells have >13,000 metastatic cells in the lung (Figure 2A, large panel). The range of primary tumor growth and varying number of metastatic cells in individual mice most likely reflects the heterogeneity of the 4T1 tumor (4, 5).

*Treatment of Tumor-bearing Mice with Transfectants Expressing MHC Class II, B7.1, and SEB Reduces Established Wild Type Metastatic Cancer.* Previously, we have shown that transfectants expressing MHC class II or B7.1 were able to reduce metastatic disease that had been established for 9-14 d (5). However, the success of this treatment was limited to small tumor burdens and did not completely eliminate spontaneous metastases. One potential reason for this limitation was that the transfectants did not co-express MHC class II and B7.1, as previous data, using a different tumor, shows that the co-expression of these molecules works synergistically (14). We have generated 4T1 transfectants which co-express MHC

class II and B7.1 as detected by indirect immunofluorescence staining (data not shown) to test this hypothesis. In addition, we have combined the MHC class II/B7.1 double transfectants with the SEB transfectants, reasoning that SEB may provide additional proliferation signals to the tumor-specific T cells activated via the MHC class II/B7.1 interaction.

The therapeutic potential of the combination vaccines was rigorously tested by treating BALB/c mice with extensively-established wild type primary and metastatic 4T1 tumors. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with $7x10^3$ wild type 4T1 tumor cells. Starting 2 wk after challenge, they were given i.p. injections of irradiated transfectants (4T1/SEB-12, 4T1/SEB-14, and/or $4T1/A^d/B7.1$ as indicated) twice a week until the day of sacrifice. Therapy was initiated at 2 wk post primary tumor challenge because spontaneous lung and LN metastases are well established by this time (5). At the time of sacrifice (6 wk after the initial primary tumor challenge), primary tumor diameters of control-treated mice (ie. mice given irradiated 4T1 cells) were comparable to tumor diameters in transfectant-treated animals, 6.5-10.5 mm and 6.2-11.2 mm, respectively. The two-tailed p-value is 0.61 when comparing the primary tumor sizes of mice treated with control cells vs. transfectant-treated mice combined. Therapy with the transfectants, therefore, does not reduce primary tumor growth.

To assess the metastatic disease, lungs from the treated mice were removed and processed to determine the number of clonogenic tumor cells. Since this therapy will be used to treat patients with established tumor, the results of this experiment have been plotted as number of clonogenic metastatic cells in the lungs

vs. tumor diameter at the start of treatment. As shown in Figure 3, administration of 4T1 transfected cells significantly reduces the number of lung metastases (Figures 3B-3D) relative to treatment with wild type 4T1 cells (Figure 3A) when primary tumor diameters at the start of treatment were <4 mm. For example, 3/16 (18.8%) mice treated with irradiated parental cells (Figure 3A) contained $<10^4$ clonogenic lung metastases in contrast to 11/23 (47.8%) mice treated with either 4T1/SEB or 4T1/$A^d$/B7.1 (Figures 3B and 3C) and 15/21 (71.4%) mice treated with a mixture of transfectants (Figure 3D). After transforming the number of clonogenic metastases to logarithmic values and analyzing using the Tukey's Honestly Significant Difference Test ($p= 0.05$) only the mixture of 4T1/SEB and 4T1/$A^d$/B7.1 (Figure 3D) treatment was significantly different from the 4T1 control-treated mice. Thus, treatment of mice carrying established primary and metastatic tumor with a combination therapy of tumor cells transfectants expressing MHC class II, B7.1, and SEB genes results in significant reduction of spontaneous metastatic tumor load, while therapy with subsets of transfectants has a less dramatic effect.

*Post-operative Treatment of Mice with Transfectants Expressing MHC Class II, B7.1, and SEB Increases Survival.* Vaccines such as the 4T1 transfectants are likely to be most useful for the treatment of disseminated spontaneous metastatic disease since primary tumors can usually be readily eliminated by surgery. Therefore, we have tested the combination vaccine in mice with established, disseminated spontaneous metastases following surgical removal of the primary tumor. Before performing this therapy experiment, we determined the appropriate time for removing primary tumors that would guarantee establishment of spontaneous

metastases. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with $7x10^3$ wild type 4T1 tumor cells. Starting 2 wk after challenge, their tumors were measured and surgically removed at one week intervals. Figure 4A shows the tumor size (mm) at the time of surgery vs. the number of days the mice survived post 4T1 tumor challenge. The average survival time of 55/58 mice was 48.9 ($\pm$ 7.4) d while the remaining 3 mice, whose tumor diameter was < 3mm at the time of surgery, lived >90 d and did not succumb to metastatic cancer. Surprisingly, all mice that died from spontaneous metastatic disease showed approximately the same mean survival time regardless of the size of the primary tumor at the time of surgery. These results demonstrate that lethal metastasis occurs as early as 2 wk after inoculation of primary tumor and that death following surgical removal of primary tumor results from outgrowth of tumor cells that metastasize early during primary tumor growth.

Based on these data, we tested the combination vaccine therapy in post-surgery mice. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with $7 \times 10^3$ wild type 4T1 tumor cells. The tumors were allowed to grow and metastasize for 3 wk, at which time the primary tumor burden was measured and surgically resected. The experiments in Figures 3 and 4A and our previously published results (5) established that at this stage and size of primary tumor the mice have very extensive disseminated metastases. At the time of surgery, the mice were divided into two groups, one group to be treated with control cells (parental 4T1 cells) and the second group to be treated with the combination vaccine (4T1/$A^d$/B7.1 + 4T1/SEB cells). The average diameter of primary tumors in

the two groups at the time of surgery was comparable (5.2 and 5.4 for control-treated vs. vaccine-treated, respectively). Therapeutic injections of irradiated cells ($1x10^6$) were started 1 wk after surgery (i.e. 4 wk after initial tumor challenge) and were administered once a week for the duration of the experiment. Figure 4B shows the survival profiles for both groups of mice. The average survival time in days for parental cell-treated and vaccine-treated animals was 40.5 ($\pm$4.0) and 48.9 ($\pm$8.4), respectively. The two-tailed p-value is 0.04 when comparing the survival time of mice treated with control cells vs. transfectant-treated mice.

## DISCUSSION

In most clinical situations primary mammary tumors are completely curable by surgery, yet approximately 33% of patients die subsequently from spontaneous metastatic disease. Metastatic disease may be clinically detectable at the time of surgery, however, micrometastatic disease is usually undetectable. To model these patterns of human breast cancer progression, we are using the 4T1 mouse mammary carcinoma. Previous studies have demonstrated that this tumor is a highly malignant tumor that rapidly and spontaneously metastasizes throughout the body in a pattern similar to human breast cancer (5). As shown in this report, 4T1 is also similar to human mammary carcinoma in that morbidity is due to outgrowth of spontaneous micrometastatic tumor cells that migrate to distant organs relatively early during primary tumor growth (Figure 4A).

Because breast cancer patients die from spontaneous metastatic disease rather than primary tumor, we have focused our immunotherapeutic efforts on treating disseminated mammary cancer. To rigorously test our cell-based vaccines, we are using mice with spontaneous, extensive, established, widely disseminated metastases. The extent of spontaneous metastatic cancer in our model is significantly more advanced than that tested in other therapy experiments. Primary 4T1 tumors which have been established for 2-3 wk in BALB/c mice typically metastasize to the LN, lungs, and livers in 86%, 79%, and 19% of mice, respectively, and the numbers of micro-metastatic cells found in these organs range between 2-57, 1-338, and 0-1, respectively (5). In addition, as the primary tumors become more established (i.e. by 4-5 wk), the incidence of metastases in the lungs, livers, and

brains has increased to 91%, 82%, and 36% of mice, respectively, and the range of metastatic cells for these organs is between 6-250,000, 7-7800, and 1-116, respectively (5). In this report, we treat mice with spontaneous metastases that have been established for 2 and 4 wk. Therapy of mice with primary tumor in place with the combined MHC class II/B7.1 and SEB vaccines is highly effective in reducing lung metastases (Figure 3) and the same combination vaccine is also effective in extending survival time (Figure 4B). Interestingly, the combined cellular therapy has no significant effect on growth of primary tumor. This observation concurs with our earlier studies using MHC class II/B7.1 vaccines alone (5) but disagrees with our results in a primary subcutaneous sarcoma system. In the sarcoma model, therapy with MHC class II/B7.1 modified tumor vaccines caused complete regression of established primary tumors in approximately 55% of the mice (14). The combination MHC class II/B7.1 plus SEB tumor cell-based immunotherapy, therefore, is very effective against metastatic mammary carcinoma, which is typically unresponsive to conventional and experimental therapies and largely responsible for patient fatality.

Superantigens, including SEB, have been previously recognized as potential reagents for up-regulating T lymphocyte responses against tumors. However, their use has been limited and they have not been combined with other factors that might optimize their therapeutic efficacy. For example, several studies describe redirected T cell activation using sAgs coupled to tumor-specific monoclonal, anti-idiotypic, or bi-functional antibodies (15-17). SEB has been administered systemically along with tumor cells and SEB DNA has been inoculated intratumorally along with cytokine

DNA to assess their ability to reduce primary tumor growth (18, 19). In addition, sAgs have been used to activate tumor-draining LN T cells ex vivo for adoptive transfer into tumor-bearing animals (20, 21). All of these approaches produce some reduction in primary tumor growth and/or decrease in metastatic lesions. However, the test settings have involved relatively small primary tumor and/or very small metastatic tumor burdens which do not necessarily mimic the clinical situation. These results, taken together with the SEB transfected tumor vaccines presented in this report, show that SEB expression alone has a modest effect on tumor progression. However, the anti-tumor effect of SEB is significantly amplified when combined with the cell-based vaccine containing additional immunotherapy molecules such as MHC class II and B7.1.

Although the mechanism by which the MHC class II/B7.1/SEB tumor cell-based vaccines regulate the outgrowth of spontaneous metastases is not fully understood, other mechanistic studies suggest that the three transfected genes are working synergistically to optimally activate both $CD8^+$ and $CD4^+$ T lymphocytes. The modified tumor cells may function directly as APC for the initial activation of tumor-specific $CD8^+$ and $CD4^+$ T cells following immunization. Previous studies demonstrate that both $CD8^+$ and $CD4^+$ T lymphocytes are involved in immunity induced by MHC class II/B7.1 vaccines (14) and that MHC class II/B7.1 modified tumor cells function directly as APC for the initial activation of tumor-specific $CD4^+$ T cells following immunization (22). Direct presentation of antigen by tumor cells is possible since tumor cell expression of MHC class II molecules in the absence of invariant chain allows for presentation of endogenously synthesized tumor

antigens by MHC class II molecules (22, 23). Because the vaccines express MHC class I, class II, B7.1, and SEB molecules, antigen-specific and co-stimulatory signals will be efficiently delivered to $CD8^+$ and $CD4^+$ T cells. Likewise, because the activated $CD8^+$ and $CD4^+$ T cells are in close proximity to each other, there should be an efficient transfer of cytokines between CTL and Th cells (see Figure 5, right-hand side).

Host-derived APC are also likely to be involved in $CD8^+$ and $CD4^+$ T lymphocyte activation. Because MHC class II serves as a ligand for sAg (10), it is very likely that any host-derived class $II^+$ cell will bind SEB. The involvement of host APC is supported by the observation that SEB transfectants alone, which do not express MHC class II, cause a modest therapeutic effect (Figure 3A vs. 3B). Furthermore, other mouse tumor models have demonstrated that both class I- and class II-restricted tumor-encoded antigen can be processed and presented indirectly by host-derived APC (22, 24, 25). Taken together, it is likely that host-derived APC, capable of migrating to LN, coordinately present SEB and tumor antigen to both $CD8^+$ and $CD4^+$ T cells (see Figure 5, left-hand side).

SEB may also enhance vaccine efficacy because it induces an inflammatory response that stimulates immunity (26). Gene transfer techniques designed to minimize sAg toxicity have demonstrated that *in vivo* expression of various sAg (SEA, SEB, and TSST-1) DNAs induces intense inflammatory responses (27). Although systemic administration of sAg (doses >500μg) typically triggers T cell release of cytokines such as TNF and lymphotoxin that leads to cachexia (11), we did not see any adverse side effects in our mice.

When a sAg, such as SEB, is co-expressed by the MHC class II/B7.1 vaccine, additional activation/proliferation signals may be delivered to the specifically activated $CD8^+$ and $CD4^+$ T cells. Because SEB binds to the sides of MHC class II molecules and the TCR while antigenic peptide binds within the MHC class II cleft (28, 29), it is feasible that the sAg, tumor antigen-specific, and co-stimulatory signals are simultaneously received by the T cells. While it is also possible that those signals are not coincident, several studies have shown that activation of T cells by SEB is facilitated/enhanced by B7/CD28 signaling (30-33). Controversy exists over the ability of co-stimulation to inhibit sAg-induced apoptosis but one report demonstrates that LPS activation of B cells prevents sAg-induced deletion (34). Regardless of the precise kinetics in which the various activation signals are delivered, coordinate delivery of the three signals clearly improves the efficacy of the vaccines to reduce spontaneous metastatic tumor outgrowth. As a result, T cell activation may be exceptionally efficient since both direct and indirect presentation occur, thus, yielding larger numbers of precisely those $CD8^+$ and $CD4^+$ tumor-specific cells that mediate tumor cell destruction.

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## FOOTNOTE

[1] *Abbreviations used in this paper: Staphylococcal aureus* enterotoxin B, SEB; superantigen, sAg; mean tumor diameter, TD.

## REFERENCES


1) Sogn, J. 1998. Tumor immunology: the glass is half full. *Immunity.* 9: 757-763.

2) Lattime, E.C., and S.L. Gerson. 1998. Gene therapy of cancer. Academic Press, San Diego. 406 pp.

3) Blankenstein, T., S. Cayeux, and Z. Qin. 1996. Genetic approaches to cancer immunotherapy. *Rev. Phys. Biochem. Pharm.* 129: 1-49.

4) Aslakson, C.J., and F.R. Miller. 1992. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res.* 52: 1399-1405.

5) Pulaski, B.A., and S. Ostrand-Rosenberg. 1998. Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines. *Cancer Res.* 58: 1486-1493.

6) Hung, K., R. Hayashi, A. Lafond-Walker, C. Lowenstein, D. Pardoll, and H. Levitsky. 1998. The central role of CD4+ T cells in the antitumor immune response. *J. Exp. Med.* 188: 2357-2368.

7) Pardoll, D., and S. Topalian. 1998. The role of CD4+ T cell responses in antitumor immunity. *Curr. Opin. Immunol.* 10: 588-594.

8) Ostrand-Rosenberg, S. 1994. Tumor immunotherapy: the tumor cell as an antigen presenting cell. *Curr. Opin. Immunol.* 6: 722-727.

9) Greenberg, P. 1991. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. *Adv. Immunol.* 49: 281-355.

10) Herman, A., J.W. Kappler, P. Marrack, and A.M. Pullen. 1991. Superantigens: mechanism of T-cell stimulation and role in immune responses. *Ann. Rev. Immunol.* 9: 745-772.

11) Marrack, P., M. Blackman, E. Kushnir, and J. Kappler. 1990. The toxicity of staphylococcal enterotoxin B in mice is mediated by T cells. *J. Exp. Med.* 171: 455-464.

12) Ranelli, D., C. Jones, M. Johns, G. Mussey, and S. Khan. 1985. Molecular cloning of staphyloccocal enterotoxin B gene in *Escherichia coli* and *Staphyloccocus aureus*. *Proc. Natl. Acad. Sci., USA.* 82: 5850-5854.

13) Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth.* 65: 55-63.

14) Baskar, S., L. Glimcher, N. Nabavi, and S. Ostrand-Rosenberg. 1995. MHC class class II$^+$B7-1$^+$ tumor cells are potent vaccines for stimulating tumor rejection in tumor-bearing mice. *J. Exp. Med.* 181: 619-628.

15) Dohlsten, M., J. Hansson, L. Ohlsson, M. Litton, and T. Kalland. 1995. Antibody-targeted superantigens are potent inducers of tumor-infiltrating lymphocytes *in vivo*. *Proc. Natl. Acad. Sci., USA.* 92: 9791-9795.

16) Penna, C., P.A. Dean, and H. Nelson. 1994. Antitumor x anti-CD3 bifunctional antibodies redirect T-cells activated in vivo with staphylococcal enterotoxin B to neutralize pulmonary metastases. *Cancer Res.* 54: 2738-2743.

17) Ochi, A., K. Migita, J. Xu, and K. Siminovitch. 1993. In vivo tumor immunotherapy by a bacterial superantigen. *J. Immunol.* 151: 3180-3186.

18) Dow, S., R. Elmslie, A. Willson, L. Roche, C. Gorman, and T. Potter. 1998. *In vivo* tumor transfection with superantigen plus cytokine genes tnduces tumor regression and prolongs survival in dogs with malignant melanoma. *J. Clin. Invest.* 101: 2406-2414.

19) Newell, K.A., J.D.I. Ellenhorn, D.S. Bruce, and J.A. Bluestone. 1991. *In vivo* T-cell activation by staphylococcal enterotoxin B prevents outgrowth of a malignant tumor. *Proc. Natl. Acad. Sci., USA.* 88: 1074-1078.

20) Inoue, M., G. Plautz, and S. Shu. 1996. Treatment of intracranial tumors by systemic transfer of superantigen-activated tumor-draining lymph node T cells. *Cancer Res.* 56: 4702-4708.

21) Shu, S., R.A. Krinock, T. Matsumura, J.J. Sussman, B.A. Fox, A.E. Chang, and D.S. Terman. Stimulation of tumor-draining lymph node cells with superantigenic staphylococcal toxins leads to the generation of tumor-specific effector T cells. *J. Immunol.* 152: 1277-1288.

22) Armstrong, T., V. Clements, and S. Ostrand-Rosenberg. 1998. MHC class II-transfected tumor cells directly present antigen to tumor-specific $CD4^+$ T lymphocytes. *J. Immunol.* 160: 661-666.

23) Armstrong, T., V. Clements, B. Martin, J.P.-Y. Ting, and S. Ostrand-Rosenberg. 1997. Major histocompatibility class II-transfected tumor cells present endogenous antigen and are potent inducers of tumor-specific immunity. *Proc. Natl. Acad. Sci., USA.* 120: 123-128.

24) Huang, A., P. Golumbek, M. Ahmadzadeh, E. Jaffee, D. Pardoll, H. Levitsky. 1995. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science.* 264: 961-965.

25) Pulaski, B.A., K.-Y. Yeh, N. Shastri, K.M. Maltby, D. Penney, E. Lord, and J.G. Frelinger. 1996. IL-3 enhances CTL development and class I MHC presentation of exogenous antigen by tumor-infiltrating macrophages. *Proc. Natl. Acad. Sci. USA.* 93: 3669-3674.

26) Matzinger, P. 1998. An innate sense of danger. *Sem. Immunol.* 10: 399-415.

27) Dow, S.W., and T.A. Potter. 1997. Expression of bacterial superantigen genes in mice induces localized mononuclear cell inflammatory responses. *J. Clin. Invest.* 99: 2616-2624.

28) Jardetzky, T., J. Brown, J. Gorga, L. Stern, R. Urban, Y. Chi, C. Stauffacher, J. Strominger, and D. Wiley. 1994. Three dimensional structure of a human class II histocompatibility molecule complexed with superantigen. *Nature.* 368: 711-718.

29) Li, H., A. Llera, D. Tsuchiya, L. Leder, X. Ysern, P.M. Schlievert, K. Karjalainen, and R.A. Mariuzza. 1998. Three dimensional structure of the complex between a T cell receptor β chain and the superantigen staphylococcal enterotoxin B. *Immunity.* 9: 807-816.

30) Krummel, M., T. Sullivan, and J. Allison. 1996. Superantigen responses and co-stimulation: CD28 and CTLA-4 have opposing effects on T cell expansion *in vitro* and *in vivo. Int. Immunol.* 8: 519-523.

31) Muraille, E., G. DeBecker, M. Bakkus, K. Thielemans, J. Urbain, M. Moser, and O. Leo. 1995. Co-stimulation lowers the threshold for activation of naïve T cells by bacterial superantigens. *Int. Immunol.* 7: 295-304.

32) Muraille, E., T. DeSmedt, K. Thielemans, J. Urbain, M. Moser, and O. Leo. 1995. Activation of murine T cells by bacterial superantigens requires B7-mediated costimulation. *Cell. Immunol.* 162: 315-320.

33) Blankson, J., and S. Morse. 1994. The CD28/B7 pathway costimulates the response of primary murine T cells to superantigens as well as to conventional antigens. *Cell. Immunol.* 157: 306-312.

34) Vella, A.T., J.E. McCormack, P.S. Linsly, J.W. Kappler, and P. Marrack. 1995. Lipopolysaccharide interferes with the induction of peripheral T cell death. *Immunity*. 2: 261-270.

**Figure 1**: Supernatants from 4T1/SEB transfectants stimulate proliferation of spleen cells. Naïve BALB/c splenocytes were co-cultered with soluble SEB starting at a concentration of 2μg/ml (■, ❑) or supernatants from parental 4T1 (▲, Δ), 4T1/SEB-12 (●, ○), or 4T1/SEB-14 (◆, ◊) transfectants in the presence (open symbols) or absence (filled symbols) of antibody specific for SEB.

**Figure 2:** Expression of SEB reduces metastatic potential and tumorigenicity of the 4T1 transfectants. Female BALB/c mice (5 mice/group) were injected s.c. in the abdominal mammary gland with $5x10^3$ parental 4T1 cells (A), 4T1/SEB-12 (B), or 4T1/SEB-14 (C), sacrificed 42 d later, and the number of metastatic cells in the lungs determined as described in the Materials and Methods. Primary tumors were measured every 3-4 d. The larger panels show the number of clonogenic lung metastases (x 1000) versus the tumor diameter (TD) at the time the mice were sacrificed. Each triangle represents an individual mouse. The smaller inset graphs show mean tumor diameter (TD, y-axis) vs. day post inoculation (x-axis). Each line represents an individual mouse.

**Figure 3:** Immunotherapy of established 4T1 tumors with MHC class II/B7.1 and/or SEB transfectants reduces metastatic disease. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with $7x10^3$ live wild type 4T1 cells. At 14 d post-parental tumor challenge, the TD was measured and the therapeutic injections started. Mice were treated i.p. twice a week until the time of sacrifice with $1x10^6$ irradiated parental 4T1 (A), 4T1/SEB (B), $4T1/A^d/B7.1$ (C), or a 1:1 mix of

4T1/$A^d$/B7.1 plus 4T1/SEB (D) cells. Mice were sacrificed 6 wk after initial 4T1 tumor challenge and the number of clonogenic lung metastases determined. Each triangle represents an individual mouse. Statistical analysis was performed using Tukey's Honestly Significant Difference test (p= 0.05).

**Figure 4:** A) Early spontaneous metastases outgrowth is responsible for mortality. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with $7x10^3$ live wild type 4T1 cells. Primary tumor burdens were surgically resected at varying times after inoculation (wk 2-5). Each point represents the survival time in days post primary tumor challenge for an individual mouse. B) Immunotherapy of established wild type spontaneous metastases with a mixture of MHC class II/B7.1 and SEB transfectants increases survival. Female BALB/c mice were challenged with live wild type 4T1 tumor as described in part A. Primary tumors were measured and surgically resected 21 d post-parental tumor challenge (↓). Mice were treated once a week starting at d 28 (↓) with i.p. injections of $1x10^6$ irradiated parental 4T1 (▲), or a 1:1 mixture of 4T1/$A^d$/B7.1 plus 4T1/SEB (●) cells.

**Figure 5:** Proposed mechanism of action by the MHC class II/B7.1 and SEB tumor cell-based vaccine. The SEB modified tumor cell (bottom) secretes sAg into the immunization site where host professional APC (left) and the MHC class II/B7.1 modified tumor cell (right) are able to bind the sAg and activate both $CD4^+$ and $CD8^+$ T cells.

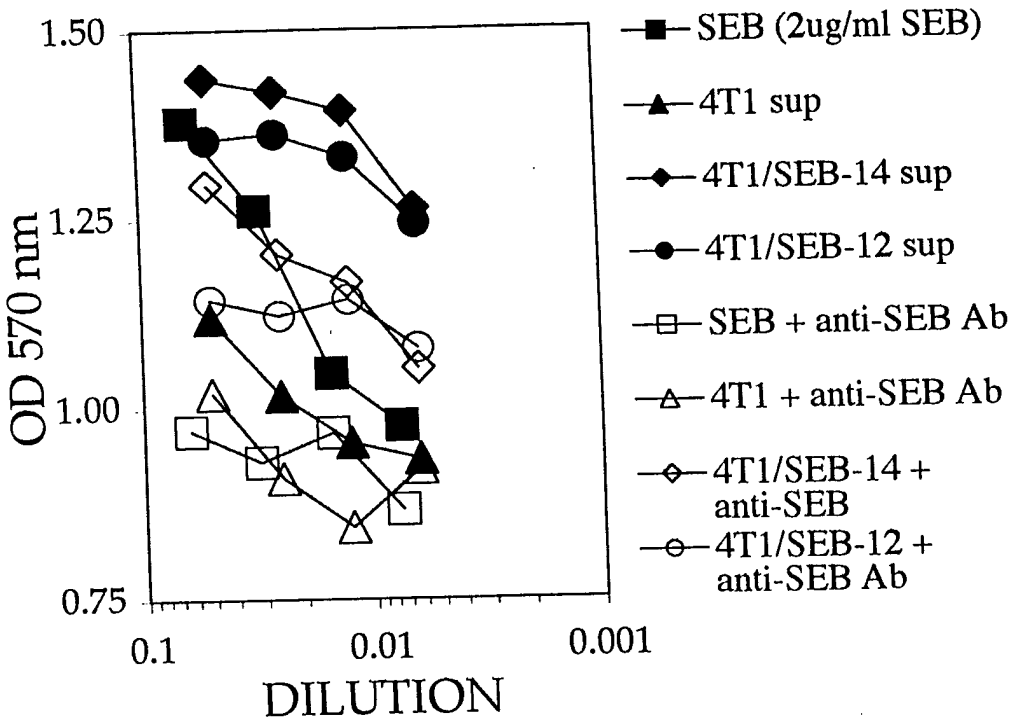
1.50
1.25
1.00
0.75
OD 570 nm
0.1
0.01
0.001
DILUTION
SEB (2ug/ml SEB)
4T1 sup
4T1/SEB-14 sup
4T1/SEB-12 sup
SEB + anti-SEB Ab
4T1 + anti-SEB Ab
4T1/SEB-14 + anti-SEB
4T1/SEB-12 + anti-SEB Ab

Figure 1

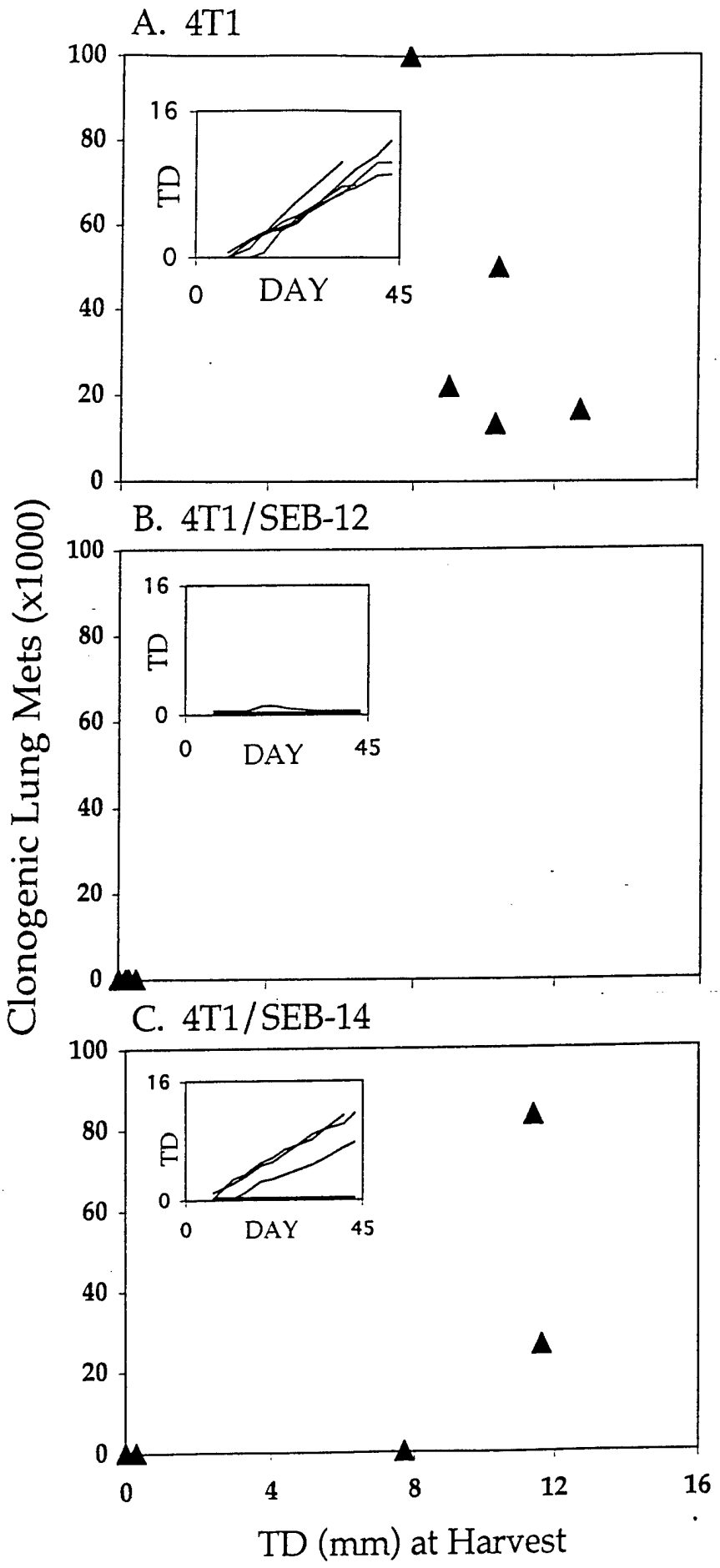
A. 4T1
B. 4T1/SEB-12
C. 4T1/SEB-14
Clonogenic Lung Mets (x1000)
TD (mm) at Harvest
TD
DAY
0
4
8
12
16
20
40
60
80
100
45

Figure 2

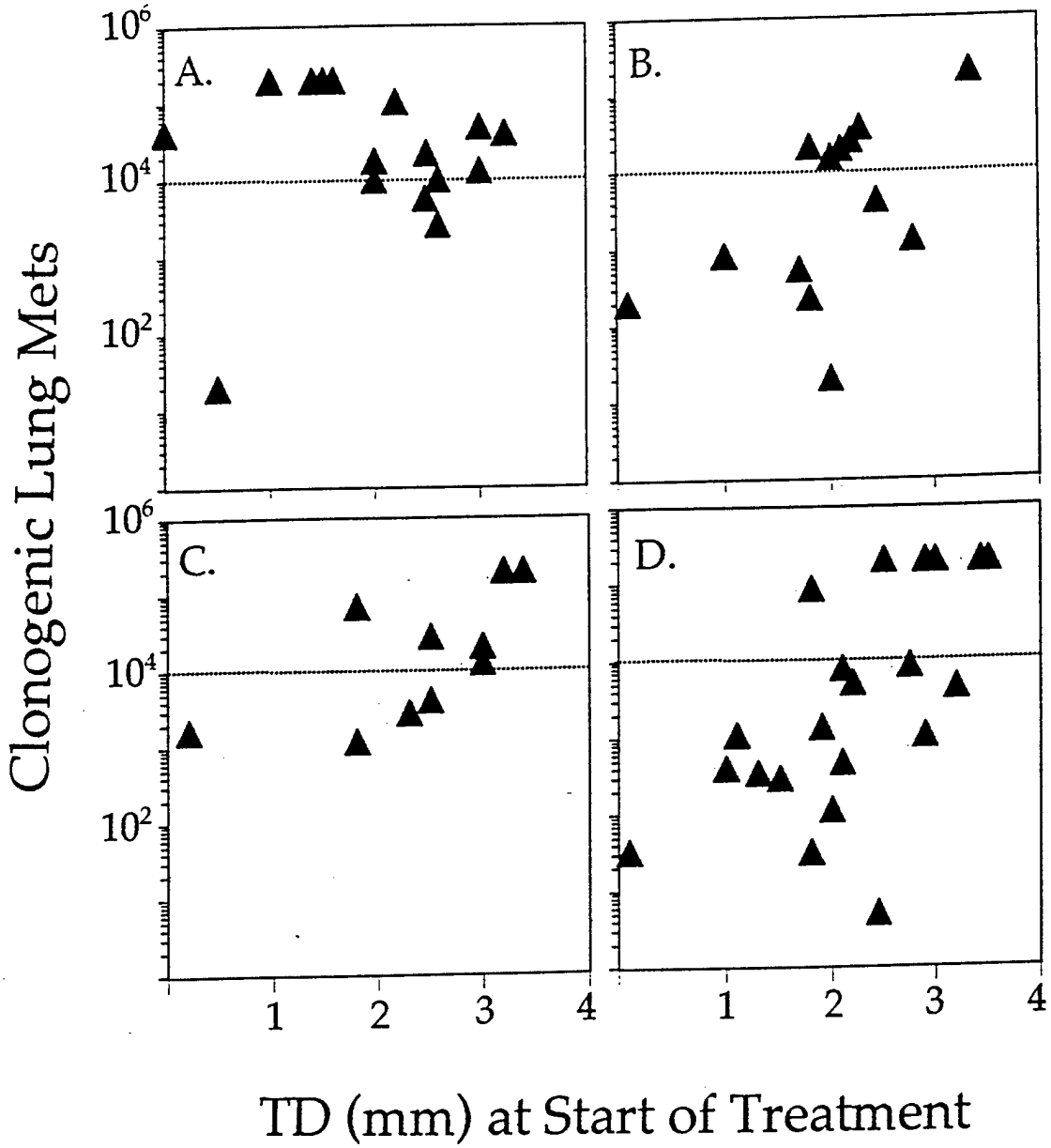
Clonogenic Lung Mets
$10^6$
$10^4$
$10^2$
A.
B.
C.
D.
1
2
3
4
TD (mm) at Start of Treatment

Figure 3

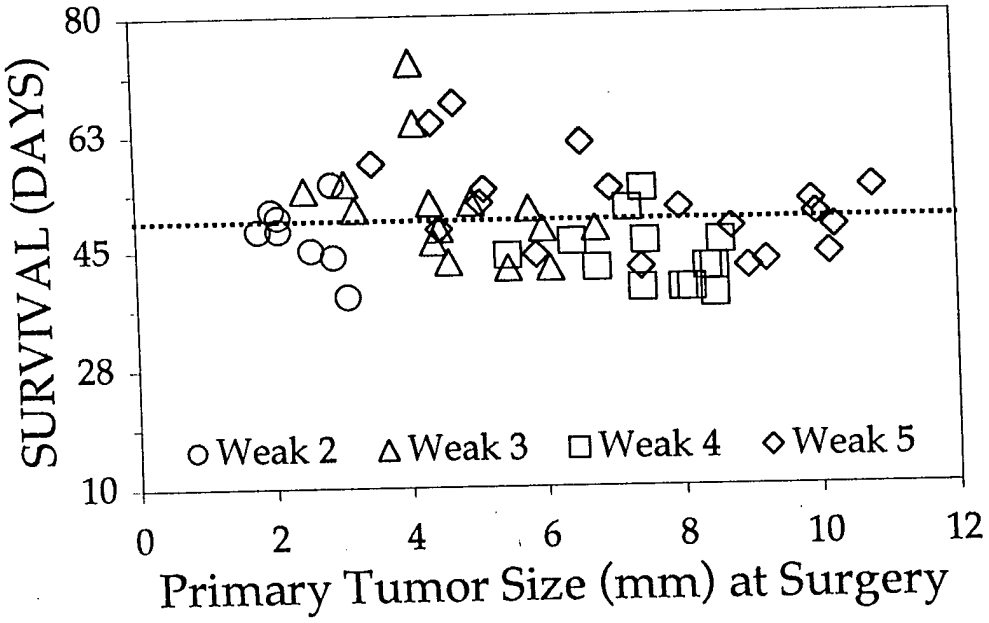
SURVIVAL (DAYS)
80
63
45
28
10
0
2
4
6
8
10
12
Primary Tumor Size (mm) at Surgery
Weak 2
Weak 3
Weak 4
Weak 5

Figure 4A

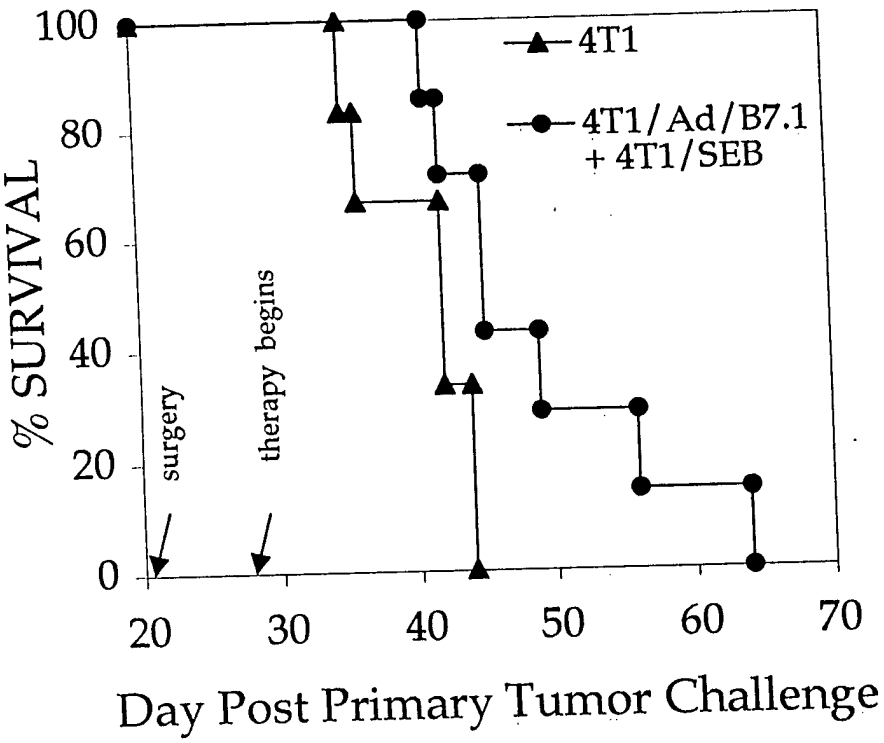
100
80
60
40
20
0
% SURVIVAL
surgery
therapy begins
4T1
4T1/Ad/B7.1
+ 4T1/SEB
20
30
40
50
60
70
Day Post Primary Tumor Challenge

Figure 4B

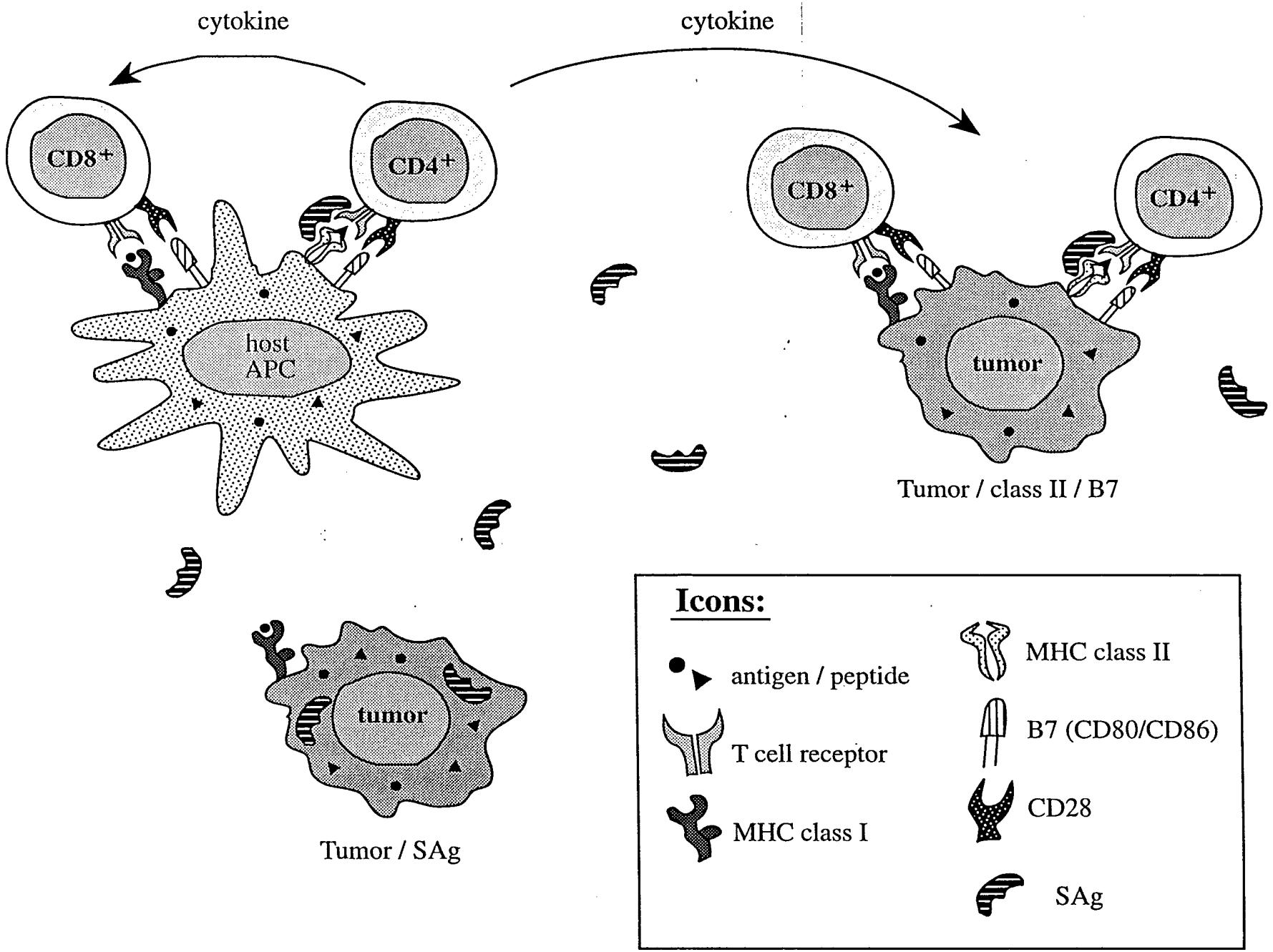
cytokine
cytokine
CD8+
CD4+
host
APC
CD8+
CD4+
tumor
Tumor / class II / B7
tumor
Tumor / SAg
Icons:
antigen / peptide
T cell receptor
MHC class I
MHC class II
B7 (CD80/CD86)
CD28
SAg

Figure 5

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**Synergy of SEB Superantigen, MHC Class II, and CD80 Genes in Immunotherapy of Advanced Spontaneous Metastatic Breast Cancer.**

Pulaski, B.A., Terman, D., Muller, E., & Ostrand-Rosenberg, S. *Dept. of Biological Sciences, University of Maryland, Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250.*

No significant improvements in the treatment of metastatic breast cancer have been developed in the last 20 years and the prognosis for women with this disease remains poor. Progress in understanding the immune response, however, has led to renewed enthusiasm for immune-based anti-cancer therapies. In previous reports, we demonstrated that tumor cell-based vaccines expressing MHC class II and B7.1 (CD80) molecules reduced experimental (i.v.-induced) and established spontaneous metastatic disease, by activating tumor-specific $CD4^+$ T-lymphocytes. We now demonstrate, using the 4T1 mammary carcinoma, that a vaccine combining MHC class II and B7.1 molecules with SEB superantigen in two distinct immunotherapeutic regimens produces an even greater reduction in spontaneous metastatic disease and significant extension of mean survival time. The therapeutic effect is particularly noteworthy because: 1) spontaneous metastatic cancer by 4T1 progresses similarly in comparison to human metastatic mammary cancer, 2) our post-operative model demonstrates that early metastatic lesions are primarily responsible for morbidity, and 3) the disseminated metastatic disease is quite extensive prior to the initiation of immunotherapy in both regimens.

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**Synergy of SEB superantigen, MHC class II, and CD80 in immunotherapy of advanced spontaneous metastatic breast cancer.**

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